Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/CA05/000517

International filing date: 06 April 2005 (06.04.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/559,439
Filing date: 06 April 2004 (06.04.2004)

Date of receipt at the International Bureau: 04 May 2005 (04.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PA 1289976

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APPLICATION NUMBER: *60/559,439*
FILING DATE: *April 06, 2004*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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PTO/SB/16 (01-04)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
HENRY L.	CLASSEN	SASKATOON, CANADA

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PRODUCING PHENOLIC ANIMAL FEEDSTUFFS AND USE FOR IMPROVING HEALTH, GROWTH AND PRODUCTION TRAITS OF ANIMALS

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Address	INDUSTRY LIAISON OFFICE
Address	Box 5000 RPO University, 110 Gymnasium Place
City	SASKATOON
State	SK
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Country	CANADA
Telephone	(306) 966-7331
Fax	(306) 966-7806

ENCLOSED APPLICATION PARTS (*check all that apply*)

☑ Specification *Number of Pages* 209
☐ CD(s), Number _____
☐ Drawing(s) *Number of Sheets* _____
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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE Douglas D. Gill
TYPED or PRINTED NAME Doug Gill
TELEPHONE (306) 966-7335

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Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
HONG YU	QIAO	SHANGHI, CHINA

Number _____ of _____

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Title: **Producing phenoic animal feedstuffs and use for improving, health, growth and production traits of animals**

Inventors: Henry L. Classen (Saskatoon, Saskatchewan, Canada)
Hongyu Qiao (Saskatoon, Saskatchewan, Canada)

Assignee: The University of Saskatchewan, Saskatoon, Canada

ABSTRACT

Sinapic acid (SA) is a secondary plant phenol metabolite found in a wide range of plants and plant products. This compound and its derivatives have been shown to be absorbed and affect digestive tract microbial ecology thereby providing benefits in terms of nutrient utilization and production performance in animal species. As disclosed in this invention, sinapic acid and derivatives, preferably sinapic acid, have antimicrobial activity and can be used in feed applications as a natural alternative to antibiotics for prophylaxis applications such as for use as a growth promotant in growing animals. As well, they can be used as digestive tract (gastrointestinal) microbial modulators in the fields of animal and human health and nutrition. At therapeutic levels, sinapic acid may be used as an antimicrobial agent to prevent intestinal tract diseases in animals and humans. Combined with the antioxidant capability of these simple phenolics, the antimicrobial activity demonstrates their further value as feed preservatives. The claim includes the usage of sinapic acid and derivatives as purified or semi-purified compounds, or as a component of value-added functional food or feed products, or included in animal products (meat, egg, milk), and their applications in animal health and production, and in human health and nutrition.

Sinapine is a choline ester of sinapic acid (4-hydroxy-3,5-dimethyoxy-cinnamic acid), the esterified form of sinapic acid and the predominant phenolic compound found in Cruciferous plants especially in the Brassica family, or in rapeseed. Sinapine could be hydrolyzed into choline and sinapic acid. A sinapine esterase enzyme system, which comprises of at least one enzyme having the carboxylic ester hydrolase activity, such as sinapine esterase, ferulic acid esterase, p-coumaric acid esterase, tannase, phenolic acid esterase, or other carboxylic esterase in

the system, could be used to hydrolyze the ester bond of sinapine into sinapic acid and choline during the rapeseed/canola meal or other feed/food processing, or after final meal processing, or as a combination technology with other forms of treatment in the process, to produce the value-added feed/ food products or higher values of food/feed grade functional protein products. In addition, the enzyme system is also being used as a feed additive in the animal diet with improved nutrient profile and enhanced nutrient utilization and performance, preferably for monogastric animal use.

FIELD OF THE INVENTION

This invention relates to the use of naturally or synthetically derived sinapic acid and its derivatives, being of physiological/biological activity thereof, to modulate the gastrointestinal microbes (microflora) of non-ruminant and ruminant animal species, including humans, and thereby improve health or promote more rapid and efficient growth in animals, and prevent disease. In addition, the present invention relates to the use of sinapic acid and its derivatives as feed preservatives. In addition, the present invention relates to the use of a sinapine esterase system which comprises of at least one enzyme with the carboxylic ester hydrolase activity, specifically hydrolyze sinapine into choline and sinapic acid to produce value-added food or feed products derived from rapeseed /canola, or used as an exogenous feed additive to promote the nutritional value of rapeseed or its derived products.

BACKGROUND

Hydroxycinnamic acid and derivatives are referred to as a group of the secondary phenol metabolites derived from the phenylpropanoid pathway in the plant kingdom. These simple phenolic acids or phenylpropanoids present as phytochemicals are at higher concentrations than the polyphenolic flavonoids and anthocyanidins in many plants (Singleton et al., 1978). Among the most widely distributed hydroxycinnamic acids in plant tissues are coumaric (PCA), caffeic (CA), ferulic (FA), and sinapic acids produced in the *Shikimate* pathway from L-phenylalanine or L-tyrosine. Their occurrence is usually in various conjugated forms resulting from enzymatic hydroxylation, *O*-methylation, *O*-glycosylation or esterification. They are found both covalently attached to the plant cell wall and as soluble forms in the cytoplasm. Sinapic acid (SA) is a free phenolic acid found in plants of the Cruciferous family, with abundant amounts found in the

Brassica genus in rapeseed, *Sinapic alba* in mustard, and Cramble. In addition, sinapic acid is predominantly bound with choline to form the esterified form of sinapine (SNP).

Sinapic acid and its derivatives, mainly the esterified form of sinapine, are traditionally considered as anti-nutritional factors (ANFs) in animal nutrition. SNP and SA are thought to be responsible for the dark color, bitter and sour taste, and astringency or phenol-like flavor of rapeseed meal (Sosulski, 1979). Therefore, they may affect palatability of rapeseed meal (RSM) especially when large proportions of RSM are added in the ration. In turn, low palatability may result in reduced feed intake and performance of growing animals (Shahidi et al., 1992, 1998), particularly in non-ruminant animal species. SA can bind with protein, such as bovine serum albumin *in vitro* (Kozlowska et al., 1990; Zandernowski et al., 1992), therefore at certain levels it has the potential to bind with proteins and digestive enzymes *in vivo*. A well-documented anti-nutritional effect of SNP is the production of fishy egg taint in eggs from some strains of laying hens due to genetically controlled low levels of trimethylamine oxidative enzymes (Pearson et al., 1981). Except for this deleterious metabolic effect of SNP, there is no in vivo evidence of toxicity, decreased nutrient utilization or performance, or other negative effects for these compounds when consumed by animals or humans.

In recent twenty years, simple phenolic acids, especially hydroxycinnamic acids and derivatives, have been known to have beneficial effects in food science and human health and nutrition. It is well recognized that some herbal medicines, which contain hydroxycinnamic acids and/or their derivatives as active components, such as mustard, Bai Jie Zi (*Sinapis alba*) and cinnamon, could be used to improve digestion and fat metabolism, as anti-inflammation and anti-fungal agents, and even for diabetes treatment (Dr. Duke's Phytochemical and Ethnobotanical Databases, 2002, Agricultural Research Service, USDA). The major active phytochemicals in these plants are sinapine (sinapic acid), sinalbin and cinnamaldehyde. Recently, health and nutrition studies have also suggested hydroxycinnamates, especially FA, CA, PCA, and chlorogenic acid, which are the most abundant hydroxycinnamic acids commonly found in human foods, have strong antioxidative activity (Cuvelier, 1992; Graf, 1992; Rice-Evans, 1996). They can act as free radical scavangers because of their hydrogen-donating ability, forming aryloxyl radicals. The stabilization of such radicals by other functional groups in the structure enhances the antioxidant

activity (US Patent: 6143543, 5908615). They may also have the anti-carcinogenous, anti-mutagenic, anti-viral (fungal), and anti-inflammatory effects. Ferulic acid has been demonstrated to possess antioxidant, anticancer, anticholesterol, antibiotic, anti-mutation and anti-inflammatory effects. (Castelluccio, et al., 1996; Fernandez et al., 1998; Saija et al., 2000). Probably due to their antioxidant potential, they are used as drugs for some human diseases, such as cardiovascular disease, atherosclerosis, hypertension, diabetes, and arthritis (US Patents: 6350473, 5008441, 6310100). Chlorogenic acid and caffeic acid are absorbed in humans and might protect against cardiovascular disease (Olthof, 2001). Ferulic acid, caffeic acid and chlorogenic acid had been found to be used as a natural food preservatives due to their antimicrobial potential on food-born pathogens, such as in food, beverage, and tea etc. (US Patents: 6022576, 6120823).

SUMMARY OF THE INVENTION

The invention disclosed herein provides a method of using low dietary levels of sinapic acid and derivatives, preferably SA (levels from 0.0005~3%), to alter the microbial ecology of animals (including humans) to the benefit of animal health, disease prevention and production performance, and also to be used as a feed preservative. Examples of sinapic acid (SA) and derivatives are preferably SA (also known as sinapinic acid), and its derivatives as salts, esters, aldehydes and alcohols. These compounds are found in a wide range of plants and plant products.

SA (4-hydroxy-3,5-dimethoxy-cinnamic acid) is a free phenolic acid found in many plants, especially in the *Cruciferae* family, *Brassica napus, Brassica campestris, Brassica rapa, Brassica juncea,* and *Sinapis alba*, and *Crambe abyssinica*. It is also found in trace amounts in other plants such as, wheat, barley, and rye. Large amounts of SA are found in the esterified form of SNP, which is a choline ester of SA. SNP contributes more than 80% of total phenolics in rapeseed (*B. napus, B. rapa*) and is mainly located in the cotyledons. SA derivatives are the conjugation products derived from cinnamic acid during plant phenol metabolism. There are four types of conjugation reactions which form esters (sinapine), aldehydes (sinapaldehyde), and alcohols (sinapyl alcohol). All of above sinapic acid and derivatives may of course be derived

through totally synthetic means, such as chemical synthesis, physico-chemical extraction and isolation, microbial transformation, etc.

It is known that simple phenolic compounds in RSM, SA and SNP, possess antioxidative activity in *in vitro* model systems (e.g. in β-carotene-linoleate system) (Wanasundara et al., 1993, 1994; Zadernowski et al., 1998). An antioxidative component in RSM was isolated and identified as 1-*O*-beta-D-glucopyranosyl sinapate, which is a glucose ester of SA, and its antioxidant property has been characterized (Wanasundara et al., 1994, 1996). The study on antioxidant activities of other hydroxycinnamic acids (chlorogenic, caffeic, ferulic and *p*-coumaric), have shown that they are all effective against the oxidation of low density lipoproteins (LDL) to cholesterol, lipid peroxidation and oxidative modification of the apoprotein B100 (Castelluccio et al., 1995). In addition, several intermediates of the general phenylpropanoid and lignin specific pathways, including hydroxycinnamates and derivatives, may have different antimicrobial activity against a range of fungus, yeasts and bacteria (Barber et al., 2000). SA has been shown to have antibacterial activity against some microbial species *in vitro* (Nowak et al., 1992; Tesaki et al., 1998, Hua et al., 1999). In addition, a sinapic acid glycoside has been claimed as medicine, particularly an anti-allergic agent (US Patent: 6242425).

It was disclosed in this invention that the antioxidant effect of sinapic acid and derivatives is consistent with the electron donating effects on the ring of the COOH—CH=CH— vs. COOH—CH_2— groups and the relationship with the number and position of hydroxyl groups in the benzene ring. At least two neighboring phenolic hydroxyl groups and a carbonyl group in the form of an aromatic ester are essential molecular features required to achieve a high level of antioxidant activity. Dihydroxylation in the 3,4 position (caffeic acid) enhances the efficacy of the latter while decreasing that of *p*-cumaric acid. Substitution of the 3-hydroxyl group of caffeic acid by a methoxy group (ferulic acid) considerably enhances the antioxidant effectiveness. Other studies on the effects of hydroxycinnamates on the induction period of autoxidizing fats have also demonstrated the order of effectiveness: CA > FA > PCA. Studies also show that the antioxidative efficiency of monophenols is increased substantially by one or two methoxy substations in positions *ortho* to the OH as in ferulic acid. Sinapic acid is more protective than ferulic acid, which is better than *p*-coumaric acid. Therefore, SA is one of the

most effective antioxidants from natural sources. It was reported (Nowak et al., 1992) that the antioxidative activity of ethanol extract containing SA and its derivatives from rapeseed, was equivalent to TBHQ (tertbutylhydroquinone) and stronger than that of BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and BHA/BHT/MGC (monoglyceride citrate).

This invention discloses that SA and derivatives, preferably SA, has *in vivo* antimicrobial activity in the digestive tract of animals (e.g. broiler chickens). Repeated experiments found that, dietary SA reduced the total short chain volatile fatty acid (VFA) (especially acetic acid) production in the hind gut (ceca) of broiler chickens (Table 1 and 2). The reduction of cecal VFA content from 10-30% was dose dependent (0.025, 0.05, and 0.10%), demonstrating that SA has strong antibacterial activity *in vivo* and can be used to modulate the fermentation pattern or microflora in the hindgut. The antibacterial property of SA has been detected for some selected bacterial strains (such as *E. coli* and *S. enteritidis*) in *in vitro* model systems (Tesaki et al., 1998), which is consistent with current *in vivo* finding. In the current invention, further microbial analysis of samples using the percentage-guanine-plus-cytosine content in the DNA demonstrated that dietary SA altered the relative abundance of bacteria in the ceca of broiler chickens (Figure 1). SA increased the relative abundance of bacteria in the ranges of %G+C 20-30 and 55-69, and decreased the amounts of microbes in the range of %G+C 40-54 in a dose dependent manner. The shift was more significant as the dosage of SA given to the broilers was increased. These data suggest that dietary SA increased the relative abundance of a small portion of *Clostridium* in the range from 20-30, and *Bifidobacterium* and *Propionibacterium* from 55-69. In contrast, SA decreased the relative abundance of *Escherichia*, *Salmonella*, partial *Bacteroides*, *Eubacterium* and *Lactobacillus* in the range from 40-54, which are the most abundant bacterial genera present in the GI tract of the chicken (Qiao and Classen, 2002). These changes, in general, support an increase in the relative abundance of bacteria generally considered beneficial, such as *Bifidobacterium* and *Propionibacterium*, and a decrease for potentially undesirable bacteria, such as *Escherichia* and *Salmonella*. Thus this shift has potential to be microbiologically and in turn nutritionally beneficial. It is well known that *E. coli* and *Salmonella* are common pathogens in animal production. Probiotics derived from *Bifidobacterium* and *Lactobacillus*, are frequently used as competitive exclusion microbial agents against these pathogens and are used as animal health promotants. Therefore, SA may be

considered to increase animal productivity via increased host health status. From this standpoint, SA may be similar in function to probiotics and may be considered a growth promotant and an alternative to antibiotics in animal production.

In contrast to the short chain volatile fatty acid production in ceca, dietary SA increased short chain fatty acid contents in the ileal digesta as SA levels increased, at least from one experiment (Table 1 and 2). This increased total volatile fatty acid production in the ileum in response t dietary SA corresponded with an increase in the apparent metabolizable energy (AME) of the diet, suggesting that increased short chain VFA production in ileum may favor more VFA absorption in small intestine and/or improve gut conditions so as to result in more efficient energy utilization. SA did not affect feed intake in broiler chickens, and actually increased feed consumption at a dose of 0.025% or lower. This is corresponds with a 6%increase in weight gain by and 2% increase in diet AME. The stimulation on the feed intake and improved nutrient utilization is dose dependent according to statistical analysis.

The antimicrobial effect of SA could be attributable to the presence of an unsaturated side chain in the compound. The efficacy in antimicrobial activity of this side chain increases with its length and the number of reactive double bonds that it contains. The presence of these double bonds enhances the reactivity of the compound after passive transport into the microbial cell. The subsequent combination effects of the dissociation of the acid moiety internal to the cell, and the accompanying presence of one or more highly reactive radicals (4-hydroxyl and two methoxyl groups), contributes significantly to the anti-microbial effect of SA. Small chemical groups that release, or by virtue of their bonding structure, readily share electrons, significantly stabilize the benzene ring's electrical charge and reduce the amount of energy needed to force reactions to take place at other sites on the ring. Relatively small electron withdrawing groups at other sites on the ring destabilize it and are therefore more easily released as highly reactive charged species or free radicals. The combination of electron releasing and electron withdrawing species on the same ring provides unique reactive properties. Other components of SA also have antibacterial action *in vitro*. The two methoxyl groups and the hydroxyl group in SA isolated from mustard seeds demonstrated antibacterial effects against *E. coli* and all of the substituents of the benzene ring were effective against *S. enteritidis in vitro*. The presence of the propenoic

group of SA was effective against *S. aureus* (Tesaki, S. et al., 1998). In the molecular structure of SA, two methoxy groups are substituted at the ortho positions relative to the hydroxyl group. Therefore, a greater antioxidant activity is also expected for this molecule.

The growth-promoting effect of SA may be similar to antibiotic growth promoters and directly come from the alteration of microbial ecology in the gut. Studies on soluble fiber and non-starch polysaccharides from various feed ingredients indicate increased fermentation in the hindgut produces energy from indigestible fibrous feed ingredients which may be positive to energy utilization, but growth-depressing effects are often observed. This is due to increased microbial growth in the hindgut, producing bacterial catabolites, such as ammonia, amine, indoles, hydrogen sulfide, secondary bile acids, and short chain fatty acids. Bacterial catabolites may be detrimental to animal health and have a negative effect on gut function (Choct et al., 1996; Mead, 1997; Langhout et al., 2000). Larger ceca are often observed in birds when fed highly fermentative indigestible feed ingredients. These points are also supported by some models using purified or semi-purified diets, either in conventional or germ-free chickens (Iji et al., 1998; Langhout et al., 2000). Therefore, reduced fermentation in the lower gut is generally considered to be beneficial. This is similar to antibiotic mechanisms of action. In addition, it is known that the suppression of the microfloral populations can improve the growth rate of the host, presumably through reduction in sub-clinical infections and reduced competition for intestinal nutrient substrates. The importance of a positive effect on gut microflora and health complements the antimicrobial activity of antibiotic growth promoters. The mode of action of SA provides a natural alternative to antibiotic, which leads to equivalent or better performances without fear of microbial resistance.

The growth-promoting effect of SA may be similar to antibiotic growth promoters and directly come from the alteration of microbial ecology in the gut. Reduced fermentation in the lower gut is generally considered to be beneficial. Increased microbial growth in the hindgut is associated with the production of bacterial catabolites, such as ammonia, amine, indoles, hydrogen sulfide, secondary bile acids, and short chain fatty acids. Bacterial catabolites may be detrimental to animal health and have a negative effect on gut function. In addition, it is known that the suppression of gut microbial populations can improve the growth rate of the host, presumably

through reduction in sub-clinical infections and reduced competition for intestinal nutrient substrates. The mode of action of SA provides a natural alternative to antibiotics which can lead to equivalent or better performance without fear of microbial resistance to antibiotics used in animal or human medicine.

It is disclosed in this invention that SA and derivatives could also be applied to animal and human health and nutrition, as an intestinal microbial modulator to facilitate a favorable microbial ecology in the gut. The bacterial modulating properties of plant extracts have been well-documented *in vitro* (Cowan et al., 1999). They can be dose-dependently bacteriostatic and/or bacteriocidal. It appears that, in this invention, a significant reduction of cecal VFA concentrations in all dietary SA levels results in a growth promoting effect through reduction in total microbial numbers and/or the change in microfloral composition in the lower gut. This finding suggests that SA could be used as a gastrointestinal microbial modulator that modifies the gastrointestinal fermentation pattern and affects microbial ecology. For some animal and human digestive tract related diseases, such as those caused by the ingestion of more soluble fiber or non-starch polysaccharides ingredients in non-ruminants and acidosis in ruminant animals, the anti-nutritive effects are usually related to excessive fermentation of carbohydrates. These compounds can be used to prevent these digestive tract diseases, through the modulation on gastrointestinal bacterial fermentation, inhibition on pathogenic bacteria growth, and facilitation of nutrient digestion and utilization. SA and derivatives occur in animal feed and human food when RSM is used as a protein source. It is known that they are absorbed and/or metabolized, and interact with the gut microflora and nutrient digestion and absorption. As discovered in the invention, they can be used at prophylaxis level to modulate the microbiological parameters and to improve the nutrient digestion and absorption process in the gastrointestinal tract of animals. Finally, SA will help to achieve a positive microflora balance in the digestive tract, facilitate nutrient digestion and absorption, prevent digestive system diseases, and improve overall health and nutritional status of the host.

Sinapine is an ester of choline and sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid), the major simple/mono phenolic compound found in many *Cruciferae* plants, especially in the Brassica family, including *Brassica napus, Brassica campestris, Brassica rapa, Brassica*

juncea, and *Sinapis alba, Crambe abyssinica,* etc. It is the esterified form of sinapic acid and contributes more than 80 % of total simple phenolics in rapeseed (including canola) meal and mainly in the cotyledons.

Simple phenolics, especially sinapine, are thought to be responsible for the dark color, bitter and sour taste, astringency or phenol-like flavor of rapeseed meal (Sosulski, 1979). In turn, the palatability problem may reduce the feed intake and performance of growing animals (Shahidi, 1993, 1998), particularly in monogastric animals, such as pigs, poultry, fish, dogs, and cats. Sinapine is also related to the deleterious organoleptic property of protein products derived from rapeseed. A significant antinutritional effect of sinapine is the production of a fishy egg taint in eggs from some strains of laying hens. Hydrolysis of sinapine by gut microorganisms results in the release of choline, which is further, metabolized to trimethylamine (TMA). Absorbed TMA can be oxidized and excreted in urine. In hens with limited oxidative capability resulting from a genetic defect of TMA oxidase the TMA is transferred to the egg yolk and causes the fishy taint (Pearson et al., 1978-1981).

Recently simple phenolic compounds, such as hydroxycinnamic acid and their derivatives, including sinapic acid and sinapine (esp. sinapic acid) have been shown to possess antioxidative activity in vitro using model system (Wanasundara et al., 1994, 1996; Pan et al., 1999). In addition, sinapic acid was shown to have antibacterial activity against some microbial species *in vitro* (Nowak et al., 1992; Teski et al., 1998). Health and nutrition studies have also suggested hydroxycinnamate, especially ferulic acid and p-coumaric acid which are the most abundant hydroxycinnamic acids commonly found in human foods, may have the anti-carcinogenous, anti-mutagenic, and anti-inflammatory effects. Therefore, sinapic acid and sinapine may have positive beneficial effects as well.

Research by the inventors using purified sinapine did not reduce feed intake and performance in broiler chickens. Sinapine improved nutrient retention as demonstrated by increased apparent metabolizable energy (AME) and fecal protein digestibility. The positive effects of sinapine may come from its nutrient moiety profile (choline) and/or its antibacterial activity of sinapic acid

moiety. Metabolic study found sinapic acid is easily absorbed when released as the free form from the sinapine molecule.

A related invention by the inventors (Qiao & Classen, 2001) disclosed that sinapic acid has antimicrobial activity in the digestive tract and therefore is a natural alternative to antibiotics either as a food or feedstuff preservative or used as a growth promotant in growing animals. Dietary sinapic acid caused a large decrease in the total short chain volatile fatty acid (VFA) (especially acetic acid) production in the hind gut (ceca) of broiler chicks, suggesting that sinapic acid has strong antibacterial activity *in vivo*. Sinapic acid increased consumption at a dose of 0.025 % and improved AME, fecal protein digestibility, and performance thereby demonstrating its beneficial nutritional effects.

Therefore, a strategy that enzymatic hydrolysis of sinapine improves the nutritional value of rapeseed meal and /or its derived feed or food products by releasing a valuable vitamin and an antibacterial compound and/or a potent antioxidant, has been followed and a new enzymatic breakdown of sinapine has been proposed. This is very different from only previously published work (Lacki et al., 1997, 1998, 1999) in Ottawa, where they were intent on decreasing sinapine content but did not perceive the nutritional value of sinapine especially its derived products after hydrolysis. It also was not aware of the importance of not having further metabolism of sinapic acid to quinones. Sinapine in rapeseed or canola meal was enzymatically transformed by an enzyme secreted by a white rot fungus *Trametes versicolor* which is a polyphenol oxidase. When canola meal was treated with this enzyme during canola crushing process, the phenolic content was decreased by 97 % under optimal temperature and pH (Lacki et al., 1998). However, from biochemical point, the products of sinapine or sinapic acid after polyphenol oxidase (oxidation) treatment should be quinone(s) or quinone derivatives, which are very biologically active compounds and more toxic than phenols. Thus, the breakdown of phenolic compounds in rapeseed products by a polyphenol oxidase treatment as the detoxification way has to be doubted and not been approved.

Because of the nutritional and metabolic effects of SA, and the nutritional value of choline as an essential vitamin, an enzymatic method has been invented to hydrolyze sinapine in feed or food

ingredients to free SA and choline. Several enzymes commercially available have been demonstrated having the carboxylic ester hydrolase activity, such as sinapine esterase, ferulic acid esterase, p-coumaric acid esterase, tannase, phenolic acid esterase, and other carboxylic esterases. Preferably ferulic acid esterase with specific carboxylic ester hydrolase activity was used for the hydrolysis of sinapine, with broad optimal temperature and pH range. It is effective and efficient with no other enzyme activities.

DESCRIPTION OF PREFERRED EMBODIMENT

Source of Sinapic acid and Derivatives

The sinapic acid and derivatives claimed in this invention will be understood to encompass, preferably as a *trans-* form, a commercially available purified compound (e.g. SA, obtained from Sigma Chemical Co., with a purity of 98.2%), a compound synthetically derived from other chemicals (e.g. SA synthesized from FA, PCA or CA), a compound extracted directly from plant materials as free acids or their derivatives as salts, esters (e.g. SNP), aldehydes, and alcohols, through various physical, chemical, and/or biological processing (e.g. isolation, filtration, evaporation, solvent extraction), or a compound prepared from enzymatic or physicochemical hydrolysis/treatment of plant material (e.g. enzymes classified in the Enzyme Classification recommendations as *E.C.3.1.* and subgroups thereof, such as carboxylic acid esterase, ferulic acid esterase, *p*-coumaric acid esterase, tannase, and phenolic acid esterase, or mild to strong acid or alkaline condition etc.), or through microbial transformation (e.g. SA derived from sinapyl alcohol). Of course, the source also includes the compounds prepared from a combination of above means.

In accordance with the present invention, the related derivatives of sinapic acid are also within the scope of the present invention. For example, the salts include salts with inorganic acids, such as hydrochloride, hydrobromide, sulfate and phosphate; salts with organic acids, such as acetate, maleate, tartrate, methanesulfonate; salts with amino acids, such as arginine, aspartic acid and glutamic acid; and salts with bases such as sodium hydroxide and potassium hydroxide. As used herein, the ester derivatives include, for example, methyl, ethyl, propyl, or isobutyl sinapic acid.

The syntheses of such compounds and derivatives are well known to those skilled in this art belonging to organic chemical or chemical synthesis.

The term plant material as used herein includes material such as seed, leaf, bark, meal or pulp produced by physicochemical processing of plants. The plant material typically comprises the residue or meal produced by physicochemical processing of plant material or the residues remaining after the extraction of seed oils, e.g. rapeseed, mustard, cereal grains, such as wheat, corn, barley, rye, and oat, sunflower, potatoes, olives, soybean, coffee, grapes, cruciferous vegetables, tobacco, herbs and such like.

The claim includes the use of sinapic acid and its derivatives as purified compounds or as a component of value-added functional food or feed products. The latter is any product whereby the concentration of sinapic acid and derivatives is above that found in its natural source as claimed above.

Use of Sinapic acid and Derivatives as Growth Promotants in Growing Animals

This invention discloses that sinapic acid and derivatives at appropriate levels in feed or food affect the microbial population of the non-ruminant and ruminant digestive tract in a positive manner and thereby improve growth related characteristics (growth rate, feed efficiency) and animal health. Sinapic acid and derivatives act as a growth promotant, and therefore are an alternative to antibiotics for growing animals, such as poultry, swine, and calf, beef cattle, sheep, fish, dogs and cats. For example, at a level of 0.025% or lower in broiler chicken diets, SA increases feed intake, improves nutrient utilization such as AME and excreta protein digestibility, while decreasing cecal microbial fermentation and altering the cecal microbial ecology. The antibacterial effect of sinapic acid may also be extended to other animal species for growth promotion purpose and improved animal health.

Use of Sinapic acid and Derivatives as Gastrointestinal Microbial Modulators in Non-Ruminant/Ruminant Species Including Humans

Sinapic acid and derivatives can be applied to animal, human and pet (dogs, cats) health and nutrition, as a gastrointestinal microbial modulator to adjust the microflora ecology, to maintain

a healthy gastrointestinal environment, and to prevent diseases. These compounds can be used to adjust internal gastrointestinal ecology and maintain a positive balance in health, through the suppression or modification of the microflora characteristics (populations and composition of microbial community). They may also be used for the prevention of acidosis in ruminant animals. Another example is the usage of these compounds to relief the deleterious or negative effects related to microbial fermentation of soluble fiber and non-starch polysaccharide feed components in the digestive tract. As disclosed in this invention, the modulation of gastrointestinal microbes resulting in an alteration of the bacterial fermentation pattern such as the reduction of VFAs in the hind gut or a favorable change in the composition of the microbial community, also have beneficial effects on host health and nutrient utilization.

Use of Sinapic acid and Derivatives as Therapeutic Agents

At therapeutic levels, sinapic acid and its derived compounds could be used as drugs for veterinary and medical purposes, in the prevention and treatment of digestive system diseases in the gastrointestinal tract. These compounds could be used to improve digestion and fat metabolism and prevent digestive tract disorders in both animals and humans. This can be achieved through reduced risk of digestive tract disease by the inhibition on the growth and colonization of pathogenic bacteria, the elimination of the chances of excessive fermentation of soluble fiber and non-starch polysaccharides resulting from the over-growth of bacteria in the hind gut; and reduce sub-clinical infections via the suppression/competitive exclusion on the microfloral populations or the modification on microbial community composition.

Use of Sinapic acid and Derivatives as Feed Preservatives

The antioxidant effect of sinapic acid and derivatives allows for their use as preservatives in feed to prevent the oxidative deterioration of fat and oxidation of other susceptible nutrients. The disclosed anti-microbial activity of sinapic acid and derivatives in this invention demonstrate that an appropriate dosage of, preferably SA and its derivatives, can be used as a natural anti-microbial-growth agent or a preservative in feeds to prevent microbial spoilage and deterioration and the spread or colonization with pathogenic organisms.

Sinapine Esterase: Source of Substrate

The substrate for sinapine esterase hydrolysis, sinapine, refers to a purified compound, synthetically derived from other hydroxycinnamic acids or their derivatives, or directly from plant isolates or extracts (e.g. from rapeseed meal) through various physical and/or chemical processing (e.g. isolation, filtration, evaporation, solvent extraction), or as its native form in rapeseed meal.

Application of Sinapine Esterase System

The hydrolysis products of sinapine by sinapine esterase is sinapic acid and choline. Due to antimicrobial activity of SA both *in vitro* and *in vivo* and the antioxidant properties, sinapic acid is being used not only as a food or feed preservative, or a native preservative without extra supplement in sinapic acid containing ingredients, but also being included in the food or feed grade products to increase their nutritional value, preferably produced from rapeseed or canola, or to produce a functional protein product with antibacterial and potent antioxidative activity which is applied in both animal and human health and nutrition. In addition, in consideration of sinapine molecule, the choline moiety, which is an essential vitamin in animal and human nutrition, and normally added in the vitamin premix of animal diets, participates in the metabolism of methyl group in both plant and animal. Therefore, an enzyme (system) with sinapine esterase (sinapine ester hydrolase) activity is used to hydrolyze sinapine, freeing the choline moiety and producing the sinapic acid thereof. The nutritional value of rapeseed or its derived products is largely increased through the increased utilization of choline profile and nutritional beneficial effects of sinapic acid in monogastric animals, and potent neutracetical impact on human health and nutrition.

Properties of Sinapine Esterase System

The sinapine esterase used for sinapine hydrolysis is an enzyme system, comprising of at least one enzyme having carboxylic ester hydrolase or sinapine esterase activity, such as ferulic acid esterase, p-coumaric acid esterase, tannase, phenolic acid esterase, or other carboxylic esterase enzymes. Such of an enzyme, may come from the isolation and purification of an plant enzyme, such as the sinapine esterase obtained from the seed during seedlings of *Brassica* plants, or from the sinapine esterase produced from genetically modified plant especially in rapeseed, or

predominantly from microbial, such as a ferulic acid esterase from *Aspergillus niger* etc. with sinapine ester hydrolase activity. Such of an enzyme is specific to hydrolysis of hydroxycinnamoyl esters without significant other enzyme activities therefore has no further activity on the transformation of sinapic acid after sinapine hydrolysis, such as activity of monophenol oxygenase, (poly)phenol oxidase, phenolic acid oxidase etc., which may oxidize sinapic acid to final products quinones or their derivatives. This is a key characteristic required of this enzyme.

Enzymatic Hydrolysis of Sinapine

The method of hydrolysis of sinapine, preferably an enzymatic treatment with the hydrolysis of sinapine into sinapic acid and choline was disclosed in this invention. In the enzyme system, ferulic acid esterase (FAE) from *Aspergillus niger* is one of the preferred enzyme classifications with a broad optimal temperature (50-60 C), and pH range (4.0-6.0) for the effective and efficient hydrolysis of sinapine under water or citric acid buffer conditions. This method has the advantage to free the choline and simultaneously produce the sinapic acid from sinapine moiety, therefore with improved nutrient profile without requirement of choline supplementation in the animal diet, and promoted neutracetical/nutritional effects of sinapic acid in animal and human health and nutrition. This enzyme (system) is effective and efficient in the hydrolysis of sinapine both in the in vitro standard sinapine stock solution and in commercial canola/rapeseed meal. After 20 minutes treatment in either the water or citric acid buffer conditions, the sinapine content was reduced by 100 % in the standard stock solution, or at least 90 % in commercial canola meal samples, respectively.

In addition, the enzyme with sinapine ester hydrolase activity can be supplemented in animal /human diets as an exogenous additive, especially in sinapine containg feed /food such as functional protein products derived from rapeseed, animal compound feed, or directly added into drinking water for supplementation in animal feeding. Such supplementation results in improved nutrient profile, beneficial effects on animal growth promotion, and on health and nutrition in both monogastric animals and humans.

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ABBREVIATIONS

AIA	Acid insoluble ash
AME	Apparent metabolizable energy
ANFs	Anti-nutritional factors
BSA	Bovine serum albumin
CM	Canola meal
CK	Creatine kinase
EtOH	Ethanol
FAE	Ferulic acid esterase
GC	Gas chromatography
GLM	General linear model
HPLC	High performance liquid chromatography
LD	Lactate dehydrogenase
RSM	Rapeseed meal
SA	Sinapic acid
SEM	Standard error of means
SNP	Sinapine
T3	Triiodothyronine
T4	Thyroxine
TCE	Trichlorethylene
VFA	Volatile fatty acid
%G+C	Percentage-guanine-plus-cytosine

1. INTRODUCTION AND OBJECTIVES

Canola meal (CM), or 'double low' rapeseed meal, which is a by-product of the canola oil extraction process, is recognized as a good quality protein supplement for animals. It has a protein content of 34-37%, a well-balanced amino acid content with especially high levels of methionine, and a high level of phosphorus (Bell, 1993). It has been demonstrated to be a cost effective feed ingredient in many areas of the world. CM is superior to its predecessor, rapeseed meal (RSM), but still trades at a discount relative to the price-setting protein supplement, soybean meal (SBM). The lower price of CM compared to SBM is a consequence of its lower protein and energy content, low and variable amino acid digestibility, and the presence of antinutritional factors (ANFs). The ANFs include glucosinolates, sinapine, sinapic acid, polyphenolic tannins, and phytic acid. The research contained within this thesis has focused on the simple phenolic compounds found in RSM or CM (referred to as RSM from this point).

Phenolics in RSM consist of simple phenols (mono-phenolics), and polyphenols (tannins). The simple phenolics are predominant in RSM and primarily refer to: 1) free sinapic acid, which is a phenolic acid; 2) sinapine (phenolic ester), which is an ester of sinapic acid and choline; and, 3) sinapic acid in an insoluble bound form. Sinapine is the predominant phenolic compound in RSM. Polyphenols in the form of tannins are found at low levels in RSM and are considered relatively unimportant despite the well-recognized negative impact of tannins in other feedstuffs such as sorghum.

The nutritional impact of sinapine and sinapic acid on animal growth and health, either in ruminants or monogastric animals, has rarely been studied. However, these simple phenolics are thought to be responsible for the dark color, bitter taste and astringency of the meal, and in turn the reduced feed intake and performance of animals consuming larger amounts of RSM (Sosulski, 1979; Shahidi et al., 1992; Naczk et al., 1998). Sinapic acid has been shown to bind with protein *in vitro* and may also do so in the meal, thereby reducing its digestibility. The most well-documented negative effect of sinapine in animal feeding is the production of fishy taint in eggs from some strains of laying hens (Fenwick et al., 1979a, 1980, 1981, 1984a, 1984b). Recently, these simple phenolic compounds have also been shown to have anti-oxidative activity and antibacterial potential *in vitro*.

Because of the perceived negative effects of simple phenolics and, in particular, sinapine, there has been considerable interest in reducing their levels in rapeseed. These efforts have included plant breeding, physicochemical methods and the application of biological treatments. However, unlike the situation for glucosinolates and phytic acid, where their contents can be reduced by plant breeding or through physical, chemical or biological processing, sinapine reduction has not been successful (McGregor, 1986; Selvaraj et al., 2000; Fleury, 2001). To date, no rapeseed cultivar low in sinapine or sinapic acid has been found, and research on techniques to remove or decrease the content of sinapine in RSM is limited, fragmentary and sometimes confusing. Basic knowledge of the physicochemical and biological characteristics of these compounds is still required. More importantly, the biological effects of these simple phenolics in animals have not been adequately investigated. These simple phenolic compounds may

have an important impact on the nutritional value of RSM but have received little attention.

The objectives of the current research were: 1) to investigate the nutritional impact of sinapine and sinapic acid on the performance, nutrient digestibility, and physiological parameters of broiler chickens; 2) to study the mechanisms of sinapine and sinapic acid digestion, absorption and metabolism; and, 3) to examine the potential to enzymatically breakdown sinapine in RSM into sinapic acid and choline. To accomplish these objectives, it was necessary to develop procedures for sinapine extraction, quantification and assessment of physicochemical properties.

2. REVIEW OF THE LITERATURE

2.1 Definition of canola

Canola is a term used to designate cultivars of rapeseed with defined characteristics which make them a source of healthy vegetable oil for human consumption. Canola's immediate forebear is oilseed rape which itself has a known history of several millennia. Rapeseed (RS) is among the world's most important oilseed crops, and is grown in many regions of the world. In Canada, it is second only to wheat in value and area planted (Shahidi et al., 1995). The term canola was derived from its designation as 'Canadian oil low acid'. Canola is the registered name for rapeseed containing less than 2% of the total fatty acids in the oil as erucic acid, and less than 30 μmoles of alkenyl glucosinolates per gram of oil-free dry matter of the seed. Therefore, canola is 'double low' rapeseed. Canola seed usually yields 40% oil and 60% meal, with the latter being the residue remaining after the extraction of the oil from canola seed, usually by the prepress-solvent process. CM arises from licensed cultivars of both *Brassica napus* and *Brassica campestris* (Bell, 1984).

2.2 Anti-nutritional factors in rapeseed or canola meal

Since the introduction of 'double low' rapeseed meal in the early 1970's, there has been a considerable amount of research on its nutrient and feed characteristics in

Canada and around the world. Included in this research has been the examination of ANFs in the meal which may limit its utilization as a protein supplement in animal feed. These anti-nutritinal factors include glucosinolates, phytic acid, simple phenolic compounds (sinapine and sinapic acid), polyphenolic tannins and fibrous components of the meal.

Glucosinolates are the most studied and well recognized ANF in RSM. In the presence of endogenous or microbial myrosinase, glucosinolates are hydrolyzed to form thiocyanates, isothiocyanates and nitriles. The isothiocyanate formed from progoitrin further rearranges in water to form 5-vinyl-2-oxazolidinethione. These products of hydrolysis, particularly oxazolidinethione, interfere with thyroid function, thus reducing growth. The nitriles also cause liver and kidney lesions (Bille et al., 1983; Bjergegaard et al., 1994; Liu et al., 1994).

Phytic acid is a strong chelating agent and affects the utilization of most polyvalent metal ions, particularly zinc and iron, by strongly binding them and thus making them unavailable for metabolism (Rutkowski et al., 1979; Wolters et al., 1993). Furthermore, phosphorus, as a component of phytic acid, is poorly available to monogastric species. Through their binding properties, phytic acid-mineral complexes are also thought to reduce meal protein and energy utilization by inhibiting nutrient digestion and absorption (Prattley et al., 1982; Knuckles et al., 1989; Newkirk and Classen, 1996; Rickard et al., 1997).

Rapeseed hull, which makes up approximately 30% of the oil-free meal, is high in indigestible fibre. The relatively high content of dietary fibre in RSM may also negatively impact the utilization of other nutrients and the quality of the meal; in this

regard it can be classified as an ANF. The dietary fiber includes low-molecular-weight carbohydrates, polysaccharides, pectin, cellulose and lignin, but also contains appreciable amounts of protein, glucosinolates and minerals (Bjergegaard et al., 1995; Andersen et al., 1997; Naczk et al., 1998).

The polyphenol tannins are a minor proportion of total phenolics in RSM and are considered to have little effect on the nutritional value of these meals (Blair et al., 1984). However, there is a requirement for basic research on tannins in CM since they are involved in the fibrous components of the hull and their accurate quantification needs to be re-evaluated.

The glucosinolate content of canola has been reduced through plant breeding and can be further decreased via physical and chemical processing. They are generally considered to have little nutritional impact when moderate levels of dietary RSM are used. Similarly, the effect of phytate can be reduced through the introduction of low phytate cultivars, or by the use of supplemental microbial phytase in the feed to enhance the utilization of phosphorus, minerals and other nutrients. However, efforts to effectively and efficiently decrease the levels of simple phenolic compounds have not been successful. A brief summary of CM ANFs is shown in Table 2.1.

Table 2.1 Generally recognized effects of anti-nutritional factors in rapeseed meal.

Contributor	Palata-bility	Binding to nutrient	Off-flavor product	Antioxidant & antibacterial	Thyroid function	Current concern
Glucosinolates	?	--	?	--	+	?
Sinapine	+	+	+	?	--	+
Sinapic acid	+	+	+	+	?	+
Phytic acid	--	+	--	--	--	+
Tannins	+	+	?	+	?	+
Dietary fiber	+	+	--	--	--	+

+: Yes.
--: No.
?: unknown or not clear.

2.3 Occurrence of simple phenolics in rapeseed

2.3.1 Occurrence of sinapine and sinapic acid

Sinapic acid (SA) (3, 5-dimethoxy-4-hydroxy cinnamic acid) is the major free phenolic acid in RSM; sinapine (SNP) is an ester of choline and sinapic acid which occurs widely in species of the *Cruciferae* family (Schultz and Gmelin, 1952, 1953). According to Clandinin (1961), sinapine was first found in an alkaloidal amine isolated from black mustard seeds by Henry and Garot (1825), and its structure was proposed by Gadamer (1897). It was first reported in seeds of *Brassica napus* and *Brassica campestris* by Clandinin (1961), and in *Crambe abyssinica* by Austin and Wolff (1968). The chemical structures of SNP and SA are shown in Figure 2.1.

Plants contain a large variety of phenolic derivatives, as well as simple phenols. These comprise flavonoids, stilbenes, tannins, lignans and lignin. All of these substances are derived from phenylalanine, and in some plants, also from tyrosine, both of which are formed by the *Shikimate* pathway (Fahey et al., 1989). Since the phenolic compounds derived from the two amino acids contain a phenyl ring with a C_3 side chain, they are collectively termed phenylpropanoids. Plants synthesize large amounts of phenylpropanoid acids, mainly cinnamic acids, which are either precursors of other families of phenolics or are present in soluble or insoluble conjugated forms as esters, amides or, rarely, as glycosides (Figure 2.2). The activity of phenylalanine-ammonia-lyase (PAL) determines the extent to which phenylalanine will be withdrawn from primary metabolism. The product of PAL activity, *trans*-cinnamic acid, is subjected to a series of hydroxylation and methylation reactions (Gross, 1981). Cinnamic acid

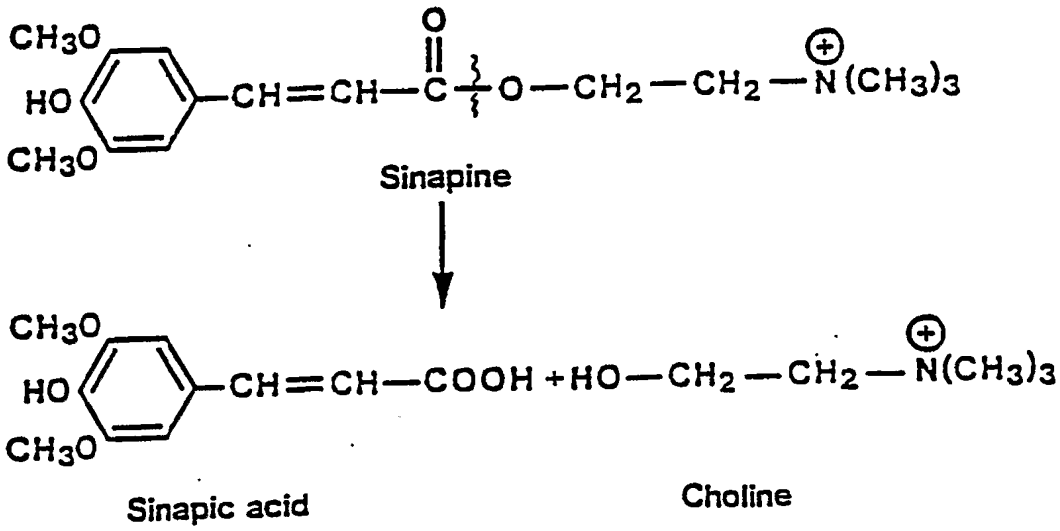


Figure 2.1 Chemical structures of sinapine, sinapic acid, and choline.

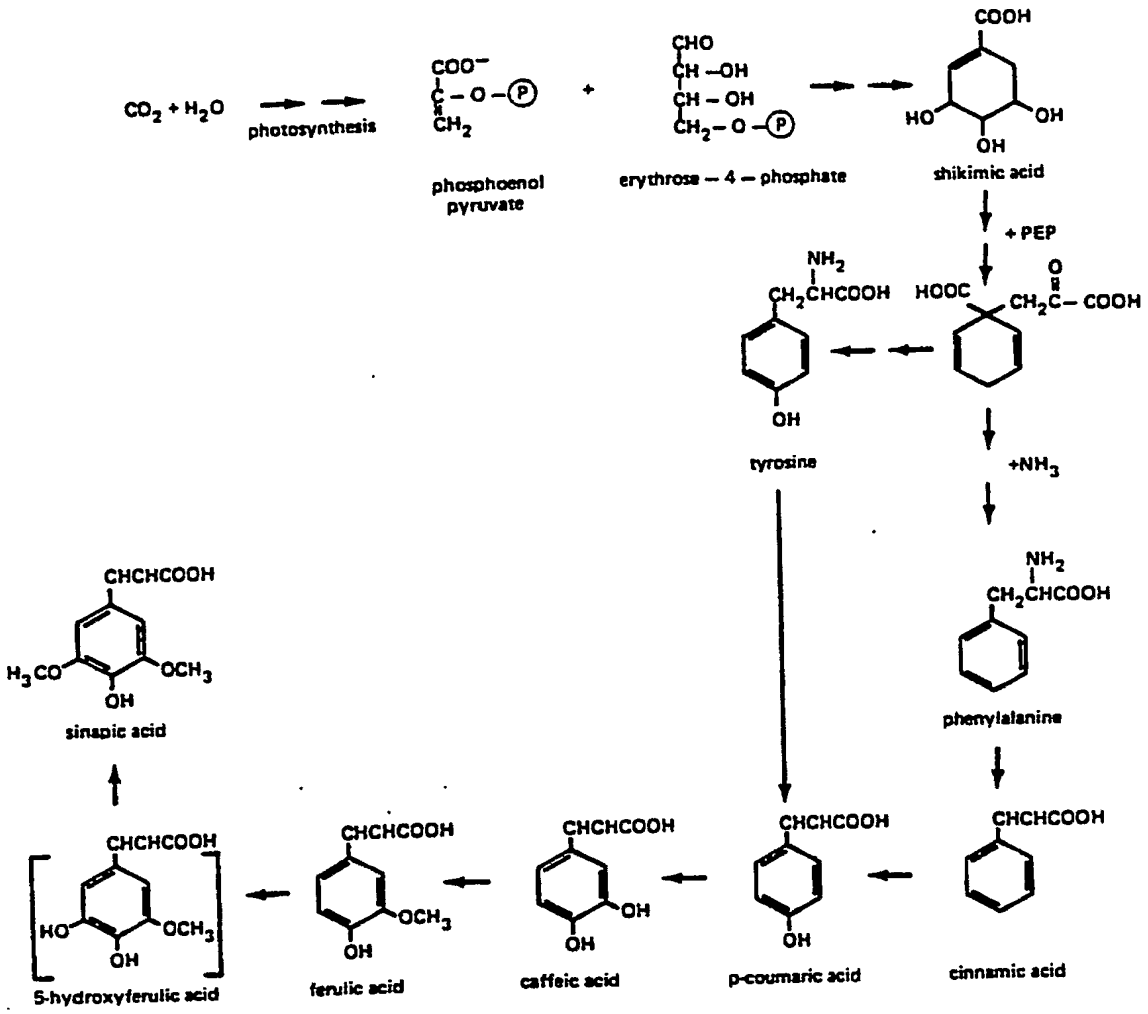


Figure 2.2 General pathway of shikimic acid synthesis and production of sinapic acid (from Fahey et al., 1989).

(unsubstituted) and the four commonly formed (hydroxy)cinnamic acids – *p*-coumaric, caffeic, ferulic and sinapic acid – enter different biosynthetic pathways, either as free acids or activated as CoA- or glucose-esters.

The formation of sinapine in plant metabolism involves conjugation reactions of phenolics. Its biosynthesis occurs in the *Phenylpropanoid* pathway and involves six enzymes which catalyze consecutive steps of transconjugation reactions, as shown in Figure 2.3. This figure shows two metabolically active periods – (1) stages of seed development and (2) stages of seed germination and seedling development – separated by seed dormancy. During the main growth phase of the developing embryo, the family-specific seed constituent, sinapine (*O*-sinapolycholine) (Schultz and Gmelin, 1952, 1953), is rapidly accumulated in the cotyledons which is dependent on PAL-mediated cinnamic acid synthesis and activation of sinapic acid by 1-sinapoylglucose:choline sinapoyltransferase (SCT) (Strack et al., 1983). The occurrence of SCT has been reported in seeds of various members of the *Brassicaceae* family (Regenbrecht and Strack, 1985) and high activities were found in seeds from the *Raphanus, Brassica* and *Sinapis* genera, all known for their high sinapine contents.

During development of seedlings, sinapine is rapidly hydrolyzed (Tzagoloff, 1963b) and the liberated sinapic acid is used for the biosynthesis of lignins and flavonoids, and choline is an important substrate in the methylation cycle.

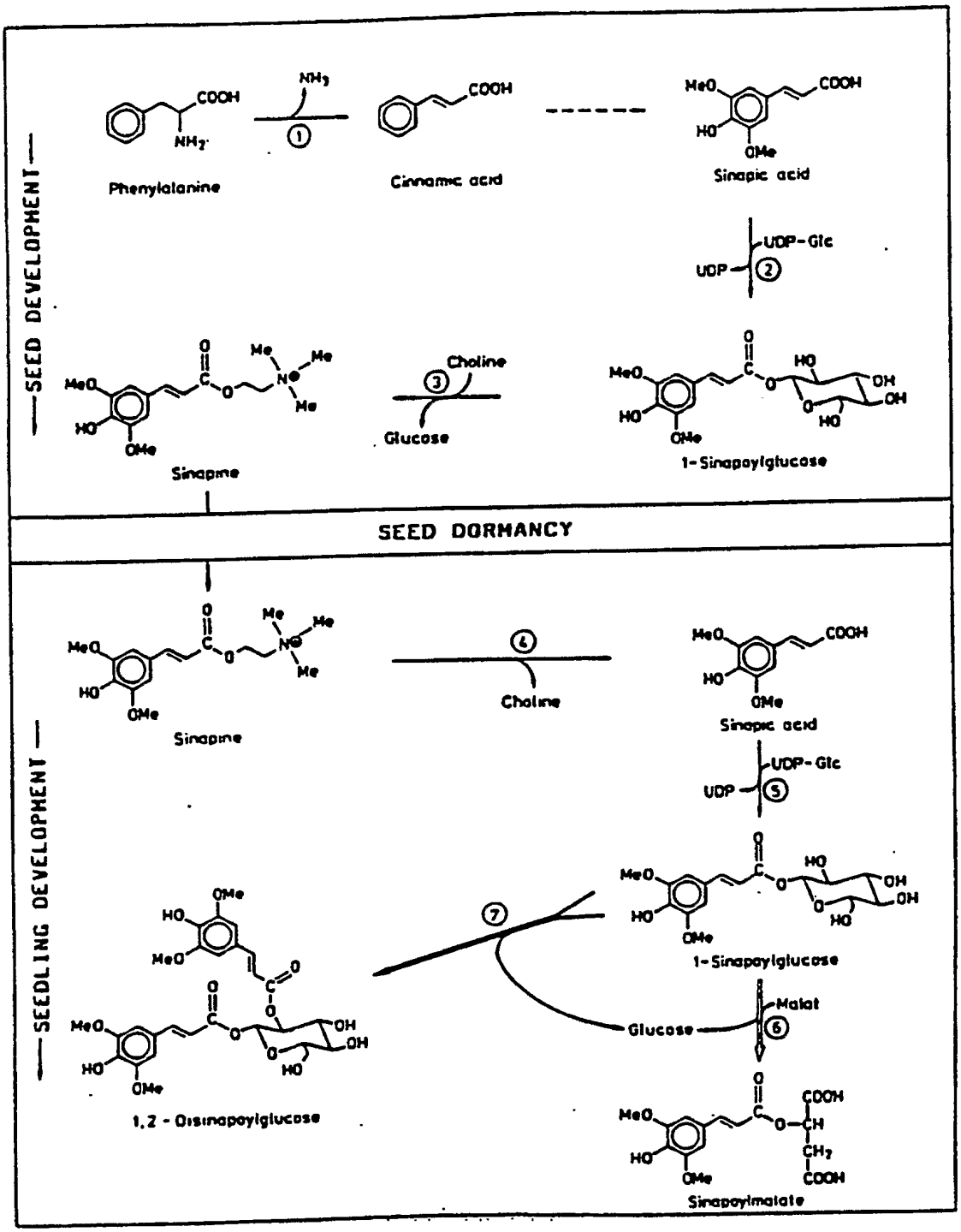


Figure 2.3 The synthesis pathway of sinapine in rapeseed (from Strack, 1983).

2.3.2 Classification and component

There is no systematic classification of total phenolic compounds in RSM due to the limited knowledge on the content, chemical composition, and function of these phenolics. The term 'phenolic' or 'polyphenol' can be defined chemically as a substance which possesses an aromatic ring bearing one or more hydroxy substituents, including functional derivatives (esters, methyl ethers, glycosides, etc.). The phenolic compounds which occur in RSM may be classified into two major groups. The predominant simple phenolics include free sinapic acid and the dominant esterified form of sinapic acid – sinapine, while hydroxycinnamic acids and their derivatives, and the insoluble bound form of phenolic acids make up the remainder (Figure 2.4). Flavonoids are in the second group and are mainly polyphenols – tannins. Tannin content ranges from 0.09 to 0.39% in defatted RS cotyledons, 0.23 to 0.54% in defatted canola cotyledons (Blair and Reichert, 1984), and 0.68 to 0.77% condensed tannins in RSM (Shahidi et al., 1989).

Rapeseed cultivars have similar levels of phenolics (Krygier et al., 1982; Kozlowska et al., 1983; Dabrowski and Sosulski, 1984; Naczk et al., 1986; Naczk and Shahidi, 1989). The simple phenolics in RS, mainly SA, exist in free, esterified and insoluble bound forms.

SA constitutes 70.2 – 85.4% of the total simple phenolic acids in RSM and free SA contributes 6.5 to 15% of the total simple phenolics present in most flours (Naczk et al., 1989). Other minor phenolic acids identified include *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, *cis*- and *trans*-ferulic, and caffeic acids. Fluorescence and mass spectrometry methods indicate cinnamic acid (including SA) can occur in *cis* and *trans* isomeric forms (Dabrowski et al., 1984). The *trans* isomers

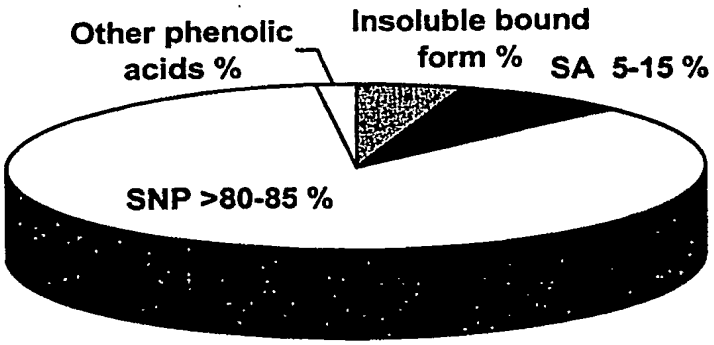


Figure 2.4 The classification and composition of simple phenolic compounds derived from RSM (SNP- sinapine; SA- sinapic acid).

predominant but the *cis* forms of SA and ferulic acids occur in most fractions. In addition, trace amounts of chlorogenic acid were found in the free phenolic acid fraction of RSM (Krygier et al., 1982; Kozlowska et al., 1975, 1983).

SNP, the esterified form of SA, is the most abundant phenolic choline ester present in RS. SNP constitutes approximately 73% of the free phenolic acids (Krygier et al., 1982) and 70-97% of the phenolic acids released from esters and glycosides of RSM (Naczk et al., 1992; Shahidi et al., 1992). Feruloylcholine, isoferuloylcholine, coumaroylcholine, 4-hydroxybenzoylcholine and 3,4-dimethoxybenzoylcholine have also been reported in RSM (Larsen et al., 1983). The total esterified phenolic acids in RS flours is much greater than in the hulls, and 99% of these are sinapic acid isomers (Krygier et al., 1982). From 91 to 93.5% of phenolic acids in RS flour are in the esterified form. Fenton et al. (1980) confirmed that SNP was the major phenolic component but found at least seven phenolic esters soluble in acetone in RSM from Midas and Echo varieties that yielded SA upon hydrolysis. Moreover, a number of other phenolic esters and glycosides have been isolated and identified in RS including methyl esters of *cis*- and *trans*- ferulic acids.

SA is the predominant insoluble bound phenolic acid in RS followed by p-coumaric and trans-ferulic acid (Kozlowska et al., 1983). SA was found to be predominant as it constituted 30 to 59% of the total insoluble fraction of phenolic acids in RS and mustard flours (Durkee et al., 1975; Kozlowska et al., 1983). Durkee et al. (1975) found that a bound form of phenolic acid in *Brassica* and *Sinapis* oilseeds was susceptible to hydrolysis by alkaline conditions and beta-glucosidase. The results indicated that SA was the prominent acid product in all species and cultivars after

hydrolysis, followed by much smaller amounts of *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid and caffeic acid. All these were liberated by alkaline hydrolysis, enzymatic hydrolysis and to some extent by acid. Hydrolysis conditions can include treatment with 2 N NaOH at room temperature for 4 hr or 2 N HCl under reflux or boiling temperature (100°C) for 30 min. SA, p-coumaric acid and p-hydroxybenzoic acids were obtained as a result of β-glucosidase hydrolysis (Durkee et al., 1975). In addition, the quantity of SA was much lower than in the ethanol-soluble fraction of RSM. Ferulic acid was quite noticeable on the hull fraction upon hydrolysis.

RSM has been reported to contain approximately 1 g of insoluble-bound phenolic acids per kg of meal (Naczk et al., 1989). However, Krygier et al. (1982) did not observe any detectable quantity of insoluble-bound phenolics in several canola flours. In another study, nine phenolic acids were identified in the fraction of insoluble-bound phenolic acids from RS flour. Tower canola hulls contained 245 mg insoluble-bound phenolics per kg of hulls. Protocatechuic acid was found to be the predominant insoluble-bound phenolic acid in Tower canola hulls while the contribution of SA to the insoluble fraction of the hull's phenolic acids was only 9.8% (Krygier, 1982). RS has also been shown to contain phenolic acid glycosides. The presence of flavonoid glycosides containing SA bound to aglycones was first reported by Durkee et al. (1973). Zadernowski (1987) found that flours of Polish RS varieties contained 0.16–0.65 μmol phenolic acid glycosides/g flour. Wanasundara et al. (1994) found that 1-*O*-β-D glucopyranosyl sinapate was the predominant glycoside in RS varieties.

In summary, SA is the dominant phenolic acid in the free, esterified and insoluble bound fractions in RSM. The contribution of SA to the insoluble-bound fraction is smaller than that found in other fractions.

2.3.3 Content

The level of simple phenolics in RSM is up to five times higher than in SBM, while the content of phenolic acids in RS flours is 10-30 times higher than that found in flours obtained from other oleaginous seeds (Kozlowska, 1991). Analytical methods for SNP have been inconsistent and this likely contributes to the variability in the reported content of SNP and related compounds in *Brassica* seeds. Moreover, the content of choline esters varies with genotype and growing environmental conditions (Bouchereau et al., 1991).

The data for SNP content in RS samples are fragmentary. A summary of reported SNP content in different RSM samples is listed in Table 2.2. The mean SNP content as thiocyanate, of 16 samples of RSM has been given as 0.91% with range from 0.58 to 1.28% (Fenwick et al., 1979a). It is independent of the level of glucosinolates (Fenwick, 1979a; Larsen et al., 1983). The content of SNP in several cultivars of *Cruciferae* crops including the *Brassica* species varied from 0.4 to 1.8% (Kerber et al., 1980). SNP is present in Canadian rapeseed meals at levels of 1.0 to 2.5% (Mueller et al., 1978) depending on growing location and cultivar. On the average, SNP (includes free SA) in RS and canola cotyledons was measured to average 2.67 and 2.85% respectively (Blair et al., 1984). A higher content of SNP was found in *Brassica napus* (1.65 - 2.26%) than in *Brassica campestris* cultivars (1.22 - 1.54%) (Mueller et al., 1978). Clausen et al.

Table 2.2 Summary of reported SNP content (%) in rapeseed meal (RSM) [1].

Author	Sample specification	Sample/Variety/ Cultivar #	Mean %	Minimum %	Maximum %	Analytical method
Fenwick et al., 1976	RSM	16 samples	0.91	0.58	1.28	TLC
Mueller et al., 1978	*Brassica napus*	4 cultivars	1.95	1.65	2.26	Reinecke salt
Mueller et al., 1978	*Brassica campestris*	4 cultivars	1.38	1.22	1.54	Reinecke salt
Fenwick et al., 1979	RSM	200 samples	1.1	0.6	1.5	Various methods
Kerber et al., 1980	Cruciferae (*Brassica*)	8 cultivars	1.1	0.4	1.8	N/A
Krygier et al., 1982	RS flour	3 varieties	0.99	0.77	1.2	GC-MS
Blair et al., 1984	RS cotyledon	7 varieties	2.67	1.4	4.0	Spectrophotometry
Blair et al., 1984	Canola cotyledon	4 varieties	2.85	2.5	3.3	Spectrophotometry
Clausen et al., 1985	*Brassica napus*	40 samples	0.84	0.62	1.06	HPLC
Clausen et al., 1985	*Brassica campestris*	9 samples	0.58	0.39	0.76	HPLC
Lajolo et al., 1991	RSM	5 cultivars	3.4	2.7	4.6	Spectrophotometry
Clark et al., 2001	RSM	5 plants	1.34	1.16	1.49	N/A

[1] Based on oil-free air dry matter.
N/A: not available.

(1985) found similar results with the *B. campestris* cultivars being lower than in the *B. napus* cultivars. The levels of SNP in this study were relatively low for both cultivars.

In summary, the SNP content of RSM varies with species, cultivar and growing condition, and range from 0.6 % to 3.0%. Sometimes the SNP content may include the value of free SA due to the limitations of analytical techniques. The evaluation of free SA content has rarely been reported. However, it can be estimated that its content in RSM may vary from 0.05 % to 0.4%.

2.4 Extraction and isolation of sinapine

Currently, there are no satisfactory solvent extraction systems that are suitable for isolation of all classes of plant phenolics or even a specific class of phenolics (Shahidi and Naczk, 1995). This is due to the chemical nature of plant phenolics which varies from simple to highly polymerized, the wide ranges of phenolics present in plant, as well as possible interactions of phenolics with proteins, carbohydrates and other seed components.

The isolation procedure of SNP from RS and the production of sinapine bisulphate was first reported by Clandinin (1961). The Clandinin procedure was used as the extraction model by several researchers (for example, Austin and Wolff, 1968) but none could obtain the same yields as described in the original paper. This is likely due to differences in extraction conditions, solvent, extraction apparatus, sample variety or cultivar, meal particle size, etc. However, all the procedures basically consisted of two steps: the isolation of sinapine as SNP thiocyanate, followed by the purification of SNP

as SNP bisulfate. In the procedure, the residual oil in the original meal is initially removed using trichloriethylene (TCE) which is very toxic and carcinogenous. Then 95 % hot ethanol is used for the extraction, and 20% potassium thiocyanate added to the extract to precipitate the SNP in its thiocyanate form. The thiocyanate form is converted to SNP bisulfate using sulfuric acid. The latter crystallization step must be conducted several times to separate the crystal salts from the solution and to obtain a purified form.

2.5 Quantification of sinapine

A standard method for analyzing SNP (or SA) in RS or canola products has not been established. Moreover, the techniques utilized to date do not give consistent results. Analysis methods used for SNP quantification are summarized in Table 2.3. To date, quantification procedures have been based on either chemical spectrophotometric or chromatographic separation methods. Spectrophotometry is a common approach used for the quantitative determination of SNP. Based on the property of absorbance of SNP or its derivatives, SNP content can be quantitatively measured directly using a spectrophotometer or indirectly after reaction with Reinecke Salt (Tzagoloff, 1963a) or titanium tetrachloride (Ismail and Eskin, 1979; Fenwick, 1981). Later, gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS) methods were also used to determine phenolic acids in RS. With the development of HPLC technology, separation and reproducible quantitative measurement of phenolic choline esters are possible using a relatively simple sample preparation procedure.

Table 2.3 A summary of quantification methods for sinapine in rapeseed products.

Quantification methods	Authors
Paper chromatography (PC)	Tzagoloff, 1963
Thin-layer chromatography (TLC)	Austin and Wolff, 1968; Krygier et al., 1982
Nuclear magnetic resonance (NMR)	Ternai and Markham, 1976; Larsen et al., 1983
Spectrophotometry (Ion-exchange UV Spectrophotometry)	Bjerg, 1984; Blair and Reichert, 1984; Clausen et al., 1982; Naczk and Shahidi, 1992; Wang et al., 1992, 1998
Gas chromatography (GC)	Krygier et al., 1982; Dabrowski et al., 1984
High performance capillary electrophoresis (HPCE)	Michaelsen et al., 1992; Bjerg et al., 1993
High performance liquid chromatography (HPLC)	Linscheid, 1980; Strack, 1983; Henning, 1983; Clausen et al., 1983, 1985; Bouchereau, 1991; Wang, 1992; Lacki et al., 1996

Among the above assay methods, spectrophotometric and HPLC analytical methods have proved to be most feasible and reliable. Both methods give practically the same results for untreated RSM. The biggest advantage of spectrophotometric methods is the short time required for the analysis of samples. A disadvantage is the non-specificity of these methods (Wang et al., 1992, 1998; Lacki et al., 1996). HPLC is more accurate than spectrophotometric methods in determining SNP and SA content, and also provides information on the presence of other phenolic choline esters. However, HPLC is relatively time-consuming. At the present time there is no standard or certified procedure for SNP or SA or phenolic analysis in RS products.

2.6 Physicochemical properties of sinapine and sinapic acid

Sinapine $[C_{16}H_{24}NO_5]^+$, 2-[[3-(4-Hydroxy-3,5-dimethoxyphenyl)-1-oxo-2-propenyl]oxy]-N,N,N-trimethylethanaminium, is a mono phenolic compound with a molecular weight of 310.38. SNP can exist in several forms of salt including chloride, iodide, or acid sulfate trihydrate - $(C_{16}H_{24}NO_5)\cdot HSO_4\cdot 3H_2O$ with the latter being freely soluble in water and hot alcohol, and very sparingly soluble in ether. It has a decomposition point at 127°C and melting point at 187°C (Merck Index, 1989). SNP is a well-defined chemical entity and its hydrolysis products are sinapic acid and choline (Figure 2.1). A characteristic feature of this group of compounds is the choline moiety has a permanent positive charge. SA is a mono phenolic acid with a molecular weight of 224.2. As derived from Sigma Chemical Co. (P.O. Box 14508 St. Louis, MO 63178 USA), it has a purity of 98% and is a light yellow color.

UV detection property. There are two major choline esters in *Brassica* plants: benzoic acid choline ester and cinnamic acid choline ester. UV spectra of solutions of the acetate salts of the aromatic choline esters reveals that the absorbance maxima for different choline esters are at different wavelengths. Most of the benzoic acid derivatives have their absorbance maxima below 300 nm and most of the cinnamic acid derivatives have an absorption peak above 300 nm (Clausen et al., 1983). The spectrum of the insoluble bound fraction exhibits bands centered at 254, 282, 319, and 384 nm (Pink et al., 1994). The absorbance maxima for SNP is 335 nm according to Clausen et al. (1985). Bouchereau et al. (1991) detected three absorbance peaks at 205, 237 and 330 nm for SNP with the latter being the maximal absorbance wavelength. Wang (1992) studied SNP bisulfate in an acetic acid / methanol solution (pH 3) and found three absorption peaks between 225 and 500 nm (230, 245 and 330 nm). In summary, the appropriate UV detection wavelength for SNP appears to be 330 nm. There are very few reports on SA, however, the UV detection maxima for SA is usually considered to be 310 nm (Wang, 1992).

2.7 Nutritional effects of rapeseed meal simple phenolics in poultry

Phenolic acids are present in all plant-derived foods and in most animal diets. These compounds have been implicated as possibly influencing the toxicological, nutritional, coloring, sensory and antioxidant properties of the food or feed with which they are associated (Stich and Rosin, 1984). Simple phenolics in RSM have been

implicated as causing anti-nutritional effects but with some specific exceptions, their exact effects are poorly understood.

2.7.1 The impact of simple phenolics on diet palatability

RS and its derived protein products are considered to have low palatablility. Malcolmson et al. (1978) described the taste of water slurries of RS protein concentrates as being bitter, astringent and unpleasant in both the raw and cooked forms. It is now known that several anti-nutritional factors such as glucosinolates and tannins can reduce the palatability of RS for animals. For glucosinolates, it is the varying properties of their breakdown products such as progoitron, goitrin, isothiocyanates, etc., rather than those of the glucosinolates themselves, which are responsible for many of the deleterious effects associated with the feeding of RS products including the palatability problem (Fenwick and Curtis, 1980). However, the level of glucosinolates is very low in recent 'double low' RS cultivars, and therefore may have little or no impact on palatability.

It is known that phenolic compounds contribute to the color of the seed coat and flavor of RS derived products. Therefore, phenolics are important factors when considering RSM as a source of human and animal grade protein, because they contribute to the dark color, bitter taste, and astringency of RS protein products (Clandinin, 1961, Sosulski, 1979; Ismail et al., 1981; Shahidi et al., 1992). As already noted, phenolic compounds in RSM are mainly simple phenolics and tannins. Tannins are a component of canola hulls and can bind with protein including salivary proteins, which causes an astringency sensation. According to Haslam (1974), only tannins with a MW ranging

from 500 to 3000 may bring about the astringency sensation. Therefore, tannins may be partially responsible for the bitter taste and astringency of RSM (Matthaus, 1998).

Astringency, recognized as a feeling of extreme dryness or puckeriness, is not confined to a particular region of the mouth or tongue, but is experienced invariably as a diffuse stimulus (Joslyn et al., 1964). According to Bate-Smith (1973), the primary reaction whereby astringency develops is via precipitation of proteins and mucopolysaccharides in the mucous secretions. Some phenolic substances present in plants cause an astringency sensation over the whole surface of the tongue and the buccal mucosa (Lea et al., 1978). Phenolic acids (esters) and tannins are both implicated in the astringency flavor of RS products (Haslam, 1988).

Aria et al. (1966) described the taste characteristics of oilseed phenolic acids as sour, astringent, bitter and /or phenol-like. SNP, the most abundant phenolic ester in RS, is a bitter tasting phenolic compound and therefore it could also contribute to the unpleasant and bitter flavor of glucosinolate-free RS products (Clandinin, 1961; Sosulski, 1979). Some studies have implicated that SNP and its hydrolysis products, SA and choline, are mainly responsible for the strong bitterness and astringency of RS flours and protein concentrates. Free choline was found in both RS flour and RS protein concentrate suggesting that some hydrolysis of SNP takes place during processing and handling of RS (Ismail et al., 1981). Since it has been reported that 0.1% choline is weakly bitter in taste (Sessa et al., 1974), free choline may also contribute to the undesirable taste of RS products. Ismail et al. (1981) used the magnitude estimation test to evaluate the bitterness of solutions containing a mixture of SA and choline chloride. They demonstrated that SA and choline chloride accounted for about 80% of the

bitterness of SNP when used at equimolar concentrations. The relative order of bitterness on an equimolar basis was SNP > SA > choline. Furthermore, they reported that 50–94% of the bitterness perceived by tasting of water slurries of RS flours and concentrates could be attributed to the bitterness evoked by SNP and free choline. These results suggest that components other than free choline, SA and SNP, contribute to the objectionable taste of RS protein products.

There are very few reports of using purified SNP in animal feeding trials. A study carried out on eight 19-day-old specific pathogen-free male mice for 11 days found that, SNP bisulfate added at a level higher than the amount of SNP of white mustard meal (2%) in casein based diet had a small impact on the nutritional value of the meal (Josefsson and Uppstrom, 1973; 1976). Results showed that the mice fed SNP bisulfate had a slower weight gain and a lower feed intake than the control group, but only the decrease in feed intake was significant. Another control containing the addition of sodium bisulfate caused no effect and there few suggested that the decrease in feed intake associated with SNP bisulfate was due to the SNP component. However, the exact amount of "high level" of SNP bisulfate added in casein diet or the equivalent inclusion level of mustard meal in the mice ration was not described in this study. Thus, the result is confusing and questionable. Austin and Wolff (1968) fed a Purina diet supplemented with 0.6% SNP bisulfate to five *Sprague-Dawley* male rats for seven days. Growth rate, feed intake, and feed efficiency were the same as for rats fed the control diet. These trials were of short duration and did not use many animals, and therefore the results may not be conclusive. In conclusion, simple phenolics do have palatability characteristics and SNP and SA have the potential to affect the palatability of RSM in some animals, but their

actual impact on animal feed intake has not been determined conclusively. Moreover, understanding the palatability effects of simple phenolics is complicated by diet dosage and purity of source, animal species, adaptation and individual variation.

2.7.2 Fishy odor egg taint

A fishy or crabby taint has been detected in chicken eggs when fish meal or RSM are used as a dietary protein supplement (Vondell 1933, 1948; Cruickshank, 1939; Gasperdone et al., 1960; Miller et al., 1972; Koehler et al., 1975; Overfield et al., 1975; Mundheim et al., 1981). In both instances it has been found that the taint is due to the presence of trimethylamine (TMA) in the yolk (Hobson-Frohock et al., 1973; Wakeling et al., 1980). This characteristic had only been noted in laying hen strains which lay brown-shelled eggs, but recently was also reported in hens that lay white shelled eggs (Horiguchi et al., 1998). Research has revealed that a metabolic defect in hens leads to the problem when they are fed specific ingredients such as RSM and fish meal (Butler, 1984) (Figure 2.5).

With the exception of fish meal, the more common ingredients of poultry diets do not contain appreciable amounts of free TMA. However, TMA can also be produced by the action of enteric bacteria on choline (March et al., 1979, 1980) which is added to layer diets as a vitamin (0.6 g or more /kg), and occurs widely in dietary ingredients both in the free form and as a component of phospholipids such as lecithins. It is also present in cruciferous oilseed meals as lecithins, which separated with the gums during processing, and as the ester of SA in the form of SNP. The release of choline from SNP by enteric bacteria has been demonstrated (Muller et al., 1978) as has the conversion of

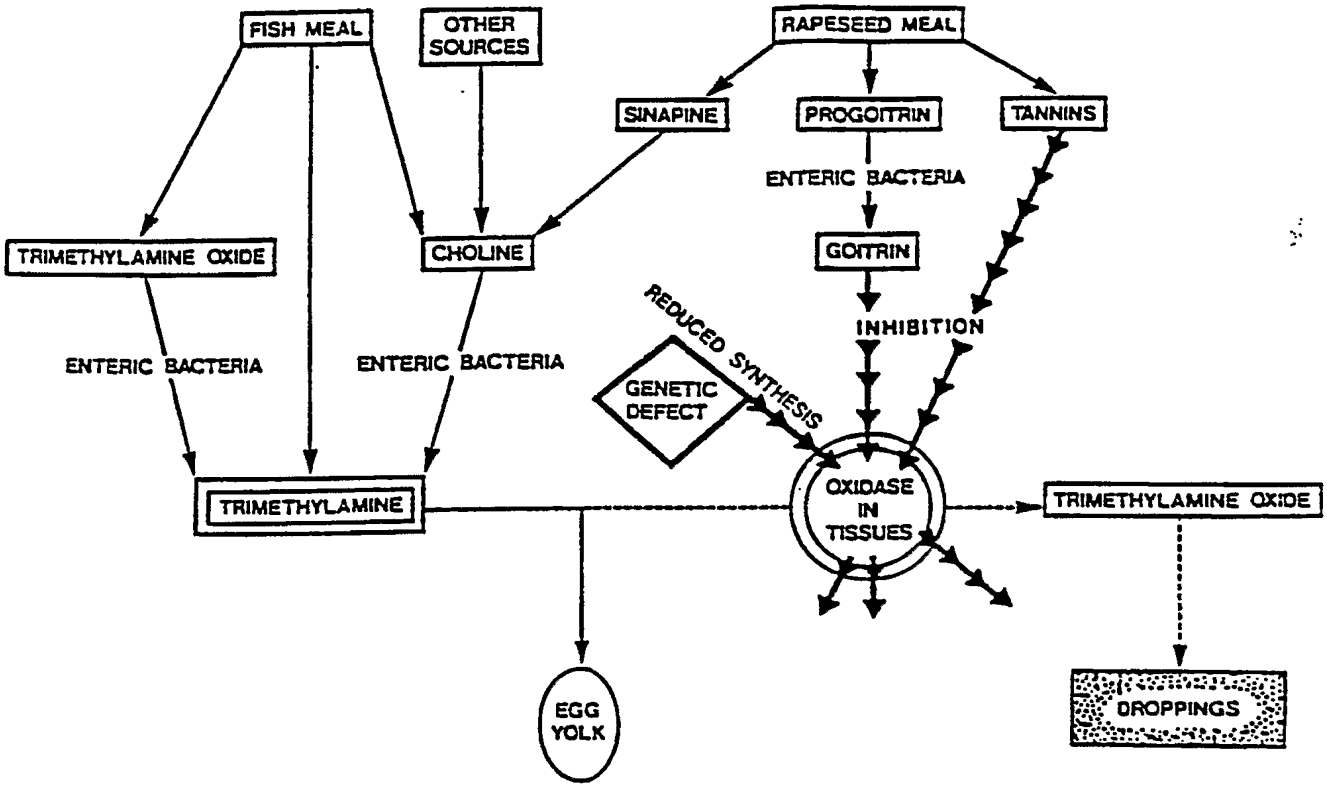


Figure 2.5 Factors involved in the production of trimethylamine (TMA) egg taint (from Butler, 1984).

choline to TMA. The amount TMA produced from SNP appears to be greater than that from an equivalent quantity of choline (Goh et al., 1979). Less choline seems to be absorbed when supplied as SNP, consequently more may be available for breakdown by the bacteria (Goh et al., 1979; March et al., 1980). It is likely that SNP hydrolysis occurs in the distal small intestine so there is less opportunity for absorption. However, Oosterwood (1975) reported that fishy flavors in eggs can be caused by TMA when the diet contains too high a level of choline.

Leeson and Summers (1977) reported that Rhode Island Red hens receiving diets containing 15% RSM produced eggs having fishy odor. This effect was accentuated when RSM contained 1.5% gums. Fishy odor was also detected in eggs produced by birds fed diets containing SBM plus 1.5% soybean (SB) gums. This demonstrates that the high choline content of both RS and SB gums also contribute to the occurrence of fishy-odor in eggs. In commercial situations, RSM invariably contains these gums. Betaine is sometimes added to diets in place of choline and it also can result in the production of TMA (Wileke, 1980).

Normally the TMA which is absorbed from the gastrointestinal tract of the fowl is rapidly converted to an odorless oxide by a microsomal enzyme (TMA oxidase) in the liver and kidneys, and then excreted in this form (Baker et al., 1963; Acara et al., 1977; Lee et al., 1982). Consistent differences between breeds and strains in the incidence of the taint when RSM is fed indicate that the problem has an important genetic aspect (Hobson-Frohock et al., 1973, 1975; Blair et al., 1975; Hawrysh et al., 1975). This was confirmed experimentally and it was concluded that a single, autosomal, semi-dominant gene was probably involved (Bolton et al., 1976). Subsequently, it was found that the

genetic factor is expressed as a deficiency of TMA oxidase and has a high heritability in males and females (Pearson et al., 1979; Pearson and Butler, 1983). Broilers generally have high TMA oxidase activities (Griffiths et al., 1980), therefore feeding RSM results in a low incidence of taint in broiler breeder eggs (Bolton et al., 1976) and no taint or off-flavor in broiler carcasses (Griffiths et al., 1980; Hawrysh et al., 1980, 1982). When the amount of TMA absorbed from the gastrointestinal tract exceeds the capacity of the hen to metabolize and excrete it, some is diverted into the developing ova and may be sufficient to taint the eggs. The average tainting threshold is about 1 μg TMA / g egg contents.

Several other constituents of *Brassica napus* and *campestris* meals have been shown to contribute to the fishy taint problem. A systematic examination of the effect of fractions of the meal on TMA oxidase activity *in vitro* revealed the presence of TMA oxidase inhibitors. These were subsequently identified as SNP and soluble tannins (Pearson et al., 1979). Further experiments demonstrated that tannins also depress TMA oxidation significantly *in vivo* (Fenwick et al., 1981). The association of a greater degree of thyroid hypertrophy with egg taint suggested that RS goitrogens might also be involved in the production of the taint. This was later confirmed to be due to a reduction in the synthesis of TMA oxidase (Pearson et al., 1978, 1979, 1980, 1981).

2.7.3 Interaction with protein

Astringency is usually related to protein binding reactions. The word astringent is derived from the Latin *ad* (to) and *stringere* (bind); thus, astringency is properly defined

as a binding reaction. Indeed, astringents in medicine and pharmacology are recognized as substances that bind to and precipitate proteins (Haslam, 1988).

Indirect evidence. Indirect studies have suggested that RSM phenolics such as SNP and SA may bind with protein. Plant biochemists have known for some time that in species containing large amounts of phenolics, extraction of functional enzymes is difficult (Van Sumere, 1975). Use of compounds such as polyvinylpyrrolidone to remove phenolics during the extraction of plant material increases yield of active enzyme preparations (Andersen et al., 1968). The potential of phenolic-protein interactions in RS is also inferred from research on protein isolation. Protein isolation procedures often require a phenolic removal step using ammonia or other bases to prevent phenolics from binding with proteins and enhance the yield of protein precipitate (Naczk et al., 1986, 1989; Shahidi et al., 1988, 1989).

Phenolic compounds are considered important factors when RSM is used as a protein source in animal feed. The ability of tannins to bind to proteins and digestive enzymes in sorghum and other ingredients is well known (Feeny et al., 1969; Mole et al., 1987; Haslam, 1989). However, for simple phenolic acids, especially hydroxycinnamates, research interest has just occurred in the recent two decades. McManus et al. (1981) postulated that simple phenolics can precipitate protein by forming a hydrophobic coating of the protein analogous to that observed for tannin-protein complexes.

Uncoupling of oxidative phosphorylation in rat liver mitochondria can be caused by various phenolics interacting with mitochondria proteins. From a biochemical point, electron transport (the oxidation of NADH and $FADH_2$ by O_2) and oxidative

phosphorylation (the synthesis of ATP) are normally tightly coupled (Voet et al., 1995). The action of uncouplers is to dissociate oxidation in the respiratory chain from phosphorylation, and this action can explain the toxic action of these phenolic compounds *in vivo*. This results in respiration becoming uncontrolled, since the concentration of ADP or Pi no longer limits the rate of respiration.

Direct evidence. There are literature reports of phenolic acids binding with proteins and enzymes. Loomis and Battaile (1966) suggested that phenols can complex with protein reversibly by a hydrogen-bonding mechanism between the hydroxyl groups of phenols and the carbonyl functionalities of the peptide bonds of proteins or irreversibly after first being oxidized to quinones, which form covalent condensations with reactive groups of protein molecules. Thus phenols could be effectively removed from H-bonded complexes with protein by adding large amounts of substances which contain groups similar to peptide. Wada et al. (1969) found that the ability to bind bovine serum albumin (BSA) correlated well with the *pKa* of simple phenols. These findings demonstrate that the hydrogen bonding between phenol and protein is stronger for more acidic phenols and that electrostatic forces play a dominant role. Zadernowski et al. (1992) extracted phenolics of RS with 80% (v/v) methanol and then separated aqueous solutions of phenolics into hydrophilic and lipophilic fractions. They found, *in vitro*, that the activities of lipase and lipoxygenase were significantly inhibited by the addition of both fractions.

Rubino et al. (1995) reported that when free SA is exposed to alkaline conditions, thomasidioic acid (TA) is formed. The transformation is complete at pH 8.5 and partial at pH 7 after 24 h exposure to 1 N NaOH (Rubino et al., 1996a). TA is a lignan, and neither

its toxicity nor beneficial properties have been investigated. However, research has been conducted on the influence of SA and TA on the rheological characteristics of RS protein gels (Rubino et al., 1996b). Interactions between these compounds and RS protein were evaluated using equilibrium dialysis. At pH 4.5, there was binding between SA and the RS protein (12S globulin) through electrostatic interactions, while at pH 7 and pH 8.5, there appeared to a hydrophobic association between TA and the protein. It is possible that the presence of either compound could decrease RS protein digestibility due to these interactions.

The formation of complexes was also investigated in model systems consisting of SA and BSA using a fluorescence spectrophotometric technique (Figure 2.6). These *in vitro* studies demonstrated that definite complex formation was favored in neutral and basic pH conditions (Smyk and Drabent, 1989).

Secondary binding. Phenolic compounds occur widely as micro constituents in plant foods and there is an increasing interest in their effects on food quality. Among the various forms of phenolic compounds, the free phenolic acids, esters, and glucosides that contain an acrylic acid group conjugated with an aromatic ring are of particular concern. These monocyclic phenolics are readily oxidized, leading to the formation of quinones, which further react to form polymers or bind to proteins and carbohydrates (Sabir et al., 1974; Van Sumere et al., 1975). Besides unfavorable organoleptic changes, oxidized phenolic compounds, can bind with essential amino acids such as lysine and methionine, forming complexes which are unassimilable in the digestive tract of animals and man (Van Sumere et al., 1975; Cheeke, 1998). This binding may lower the nutritional value of RS products (Kozlowska et al., 1975; Sosulski, 1979).

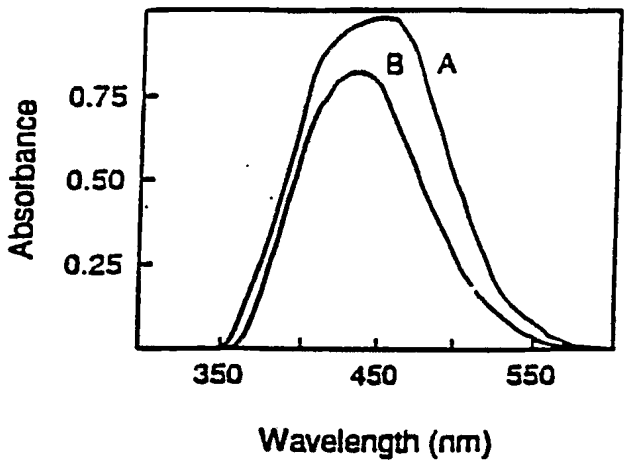


Figure 2.6 The fluorescence spectra of sinapic acid (A) and sinapic acid-bovine serum albumin (B) (adapted from Kozlowska et al., 1990).

Exposure to oxygen is necessary for oxidation of plant phenols (e.g. plant surface) and it is likely that they will interact with plant proteins at that time (Waterman et al., 1994). Products of enzymatic and nonenzymatic oxidation of phenolics in seed, meals or flours may readily react with the ε-NH_2 group of lysine and CH_3S group of the methionine of enzymes and other proteins to form complexes which are undigestible by monogastric animals (Rutkowski et al., 1977). Pierpoint (1969) has elucidated the mechanism of chlorogenic and caffeic acid binding to the ε-amino group of lysine or thiol group of cysteine. Polyphenol oxidase catalyzes the conversion of phenolic acids such as caffeic acid and chlorogenic acid to quinones. The quinones are very active, react non-enzymatically to polymerize or form covalent bonds with amino, thiol and methylene groups, as shown in Figure 2.7 (Cheeke, 1998). However, questions remain in this area, including if SA which is bound to RS protein in the meal can disassociate and be digested in the animal's gastrointestinal tract, and if quinone formation can occur during rapeseed meal processing or normal handing.

Other evidence. There is also evidence against simple phenolics binding to protein and thereby reducing meal quality. Dabrowski et al. (1984) found that simple phenolic compounds usually do not bind as extensively with proteins as polymeric tannins. There are also few reports of antinutritional effects on protein utilization in animal feeding. Rats consuming diets containing up to 4% free phenolic acids showed no change in apparent digestibility of protein, suggesting that there was little interaction with dietary protein or digestive enzymes at these levels (Hurrell et al., 1982; Jung et al., 1983). Certain studies have demonstrated a generally favorable response to phenolic acid-containing extracts. A mixture of phenolic acids containing caffic, *p*-coumaric,

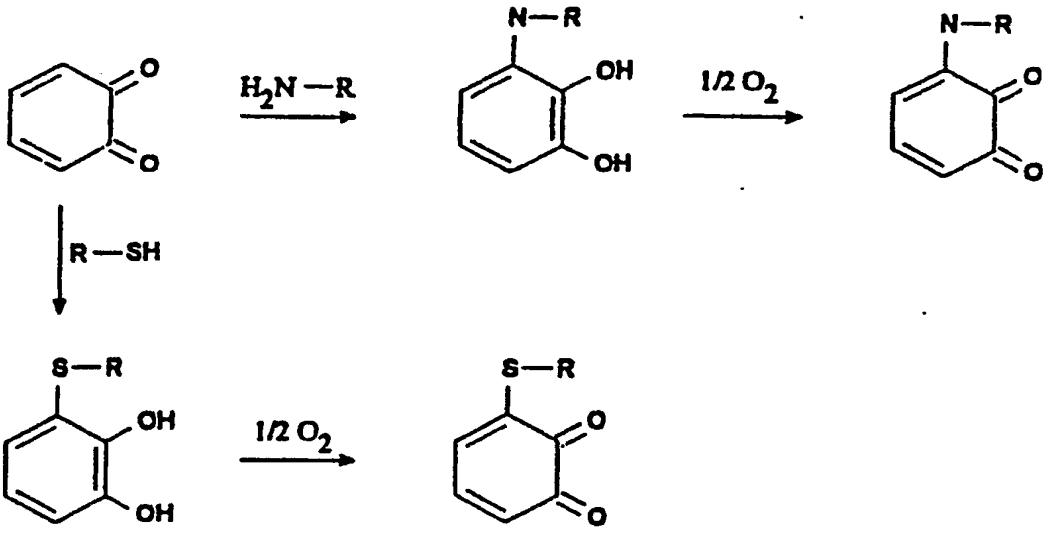


Figure 2.7 Reactions of quinones with amines or sulfur derivatives (Cheeke, 1998)

ferulic, vanillic, protocatechuic, *p*-hydroxybenzoic, and *m*-hydroxybenzoic acids equivalent to the amount of each acid contributed by 5% dietary distillers' dried solubles resulted in significant increases in growth rate of chicks (Dixon et al., 1970). To date, there is no *in vivo* evidence of the existence of binding between the simple phenolics and proteins, as well there is no direct evidence of interactions between these compounds and other nutrients.

2.7.4 Antioxidant and antibacterial activities of simple phenolics

Many preservatives are added to food and feed as antioxidants or antimicrobial agents. Phenolic compounds are known to possess both of these properties. Sinapic acid is a derivative of cinnamic acid, and the presence of a –CH=CH-COOH component in the molecule ensures better antioxidant activity of the molecule in comparison with benzoic acid derivatives possessing only a –COOH group (Cuvelier et al., 1992). In fact, sinapic, ferulic, and p-coumaric acids, all cinnamic acid derivatives, were found to be more active than benzoic acid derivatives such as procatechuic, syringic, and vanillic acids. This may be due to participation of the double bond (-- HC=CH--) in stabilizing the aryloxy radical by resonance. Other studies (Cort, 1974; Pokorny, 1987) have shown that the antioxidant efficiency of monophenolic compounds was increased substantially by substitution of electron-donor alkyl or methoxy groups which stabilized the aryloxyl radical. In the molecular structure of SA, two methoxy groups are substituted at the ortho positions relative to the hydroxyl group. Therefore, a greater antioxidant activity is expected for this molecule. Esterification of the acid group with another bulky compound may further enhance its activity. An antioxidative component in RSM was isolated and identified as

1-O-beta-D-glucopyranosyl sinapate, which is a glucose ester of sinapic acid, and its antioxidant property has been characterized (Wanasundara et al., 1994, 1996). The antioxidant activity of a crude ethanol extract of RSM on canola oil using a β-carotene/linoleate model system has been reported and the activity of the extract was equivalent to TBHQ (2-tert-butylhydro-quinone) and stronger than that of BHA (butylated hydroxy-anisole), BHT (butylated hydroxytoluene) and BHA/BHT/MGC (monoglyceride citrate) (Wanasundara, 1993, 1994). However, the impact of the antioxidative effect of these components on animals has never been evaluated.

Hydroxycinnamates, which are widely distributed as secondary metabolites in plants, exhibit a considerable antimicrobial effect under appropriate conditions. Gupta et al. (1976) found that naturally occurring ethyl *p*-methoxycinnamate inhibited growth of selected molds at concentrations of 10-50 ppm. Later Baranowski et al. (1980) reported that *p*-coumaric and ferulic acids at certain levels increased the lag phase of *Saccharomyces cerevisiae* or resulted in complete inhibition. Baranowski et al. (1982, 1984) examined the effectiveness of alkyl esters of six hydroxycinnamic and cinnamic acids (so-called alkacins), as well as free acids including SA, in inhibiting the growth of *Pseydomono flurorescens*. They found all free phenolic acids were largely ineffective at 400 ppm, on the other hand, alkyl esters of *p*-coumaric and caffeic acids had a more inhibitory effect than alkyl esters of *p*-hydroxybenzoic acid at 125 ppm. Later, they demonstrated fair to very good antimicrobial activities of alkacins on a wide range of bacteria *in vitro*. In a study conducted by Wada et al. (1969), the values of *logK* binding constant with BSA for 23 phenols was generally correlated with *pKa*, and *in vitro* bacteriostatic activity against *Staphylococcus aureus 209 P* of uncoupling oxidative

phosphorylation in mitochondria. The relationship of *logK* to bacteriostatic activity and to action of uncoupling phosphorylation of phenols were generally simple and linear. It is possible that non-specific protein bindings cause these actions of phenols. Weinbach and Garbus (1965) reported that various phenols, which uncouple oxidative phosphorylation in isolated mitochondria, produce the effect by interacting with mitochondrial protein (also see Chapter 2.7.3).

The bactericidal properties of isolated and fractionated phenolic compounds of RS were investigated *in vitro* (Nowak et al., 1992). Among the investigated groups of compounds, phenolic acids and especially SA, were found to be the most active. Antibacterial properties were determined for selected bacterial strains (*G-: E. coli 204, 205, Enterobacter aerogenes T32, R24, Pseudomonas fluorescens 87, 182; G+: Bacillus subtilis 96, B. cereus 210, Streptococcus lactis 153, S. cremoris 2*). SNP had no influence on the investigated bacteria strains. More recently, another *in vitro* test indicated that the two methoxyl groups and the hydroxyl group in SA derived from mustard seed were effective against *E. coli* and all of the substitutes of the benzene ring were effective against *S. enteritidis*. The presence of the propenoic group of SA was effective against *S. aureus* (Tesaki et al., 1998). Although the above research was completed under *in vitro* conditions, it still suggests that SA might have antibacterial activity in the gastrointestinal tract of animals.

Phenolic acid absorption and metabolism relates to gut microflora. With regard to the nutritional effects and metabolism of plant phenolics in animal species, mammals have been most thoroughly studied. Although there is no direct study on the absorption of SA, indirect evidence for a substantial absorption of monomeric plant

phenols has been obtained. A study in rats investigating the mucosal uptake of radioactively labeled cinnamic acid as a model substance in the rat jejunum, demonstrated the involvement of a Na^+-dependent, carrier-mediated transport process in the uptake of cinnamic acid and structurally related substrates such as ferulic acid across the brush border membrane of rat jejunum (Wolffram et al., 1995).

A direct study on the metabolism of SA and related compounds in rats (Griffiths, 1969) found SA and several secondary metabolites of SA in the urine, disclosing the substantial absorption of SA in the digestive tract. Other studies have suggested that ingested phenolics are absorbed as is while portions might be altered by digestive tract microorganisms before absorption. Mammalian excretion products of plant phenolics are often the result of concomitant action of both tissue-localized enzymes and reactions carried out by the gastrointestinal microflora. The two excretion pathways for foodstuff-derived phenolics are via urine and bile (Barz et al., 1985).

Digestive tract microorganisms are known to metabolize several phenolic acids (Booth and Williams, 1963). Caffeic acid (3,4-dihydroxycinnamic acid) was found to be reduced and dehydroxylated when incubated with intestinal microorganisms. Furthermore, several phenolic benzoic acid derivatives containing a free *p*-hydroxyl group were decarboxylated by rat caecal microflora (Scheline, 1966). It was also found that some compounds containing a methoxyl group underwent demethylation. Scheline (1968) orally administered caffeic acid (100 mg/kg diet) to rats. The results indicated the m-hydroxyphenylpropionic acid was a major urinary metabolite from an intestinal origin. In addition, other evidence demonstrated that the intestinal microflora had the ability to affect a number of metabolic reactions with not only cinnamic acid, but also with

phenolic phenyl acetic, and phenylpropionic acids. This study confirmed the dehydroxylation and reduction of caffeic acid as found previously and showed that intestinal decarboxylation was an important reaction for many phenolic acids which involved multiple microorganisms and enzymes. The literature demonstrates that phenolic acids can go through diverse transformations by the gut bacteria.

The only direct study of the in vivo metabolism of SA used the rat as a model animal (Griffiths, 1969). This research demonstrated the involvement of the intestinal microflora in the *p*-dehydroxylation, demethylation, and reduction of the SA. Feeding SA to rats resulted in the urinary excretion of several secondary metabolites including 3-hydroxy-5-methoxyphenylpropionic acid, dihydrosinapic acid, 3-hydroxy-5-methoxy-cinnamic acid as well as unchanged SA. Secondary metabolites in urine accounted for 26.5% of the SA administered. In the same study, sinalbin, a SA conjugate present in mustard was metabolized to free SA and 3-hydroxy-5-methoxyphenyl-propionic acid. All these metabolic studies paid much attention on the metabolic fate of SA in rats. However, how the phenolic acids affect the metabolism of intestinal bacteria, and whether these phenolics have antibacterial activity in the gut, have received no attention.

2.8 Comprehensive methods of removal of sinapine from rapeseed meal

Despite the current availability of much improved cultivars of RS, there are still several anti-nutritional factors which may limit the use of RSM, particularly for pig or poultry feeding. SNP (including SA) has been suggested to be a major factor that limits

RSM utilization. This has stimulated significant research on methods to reduce the level of SNP and SA in RS.

2.8.1 Plant breeding

Plant breeding may be the most effective method of complete elimination. However, to date, no rapeseed cultivar with a low content of SNP and other phenolic choline esters has been found. A so-called triple low RS cultivar with low contents of erucic acid, glucosinolates and tannins has been developed. However, there were no apparent benefits of triple low RS in pig diets as compared to double-low varieties (Agunbiade et al., 1991). Although SNP is normally considered to be part of the glucosinolate complex, it is evident that the reduction of glucosinolates by breeding is not paralleled by a reduction in the level of SNP (Josefsson et al., 1976; Hobson-Frohock et al., 1977). Selection for low aromatic choline esters in RS is difficult because of the complexity of this type of compound. However, traditional breeding and genetic engineering techniques are currently being used to reduce the content of aromatic choline esters and SA in *Brassica* crops (Selvaraj et al., 2000; Fleury, 2001).

2.8.2 Physical and chemical treatment

It may be possible to detoxify RS during processing using physical and chemical treatments. Dehulling may be the most simple and efficient way in promoting the nutritional value of RSM or CM by decreasing the high fiber component. However, it is difficult to accomplish this goal due to relatively small seed size (Sosulski et al., 1981; Baudet et al., 1983; Diosadyet al., 1986). The mechanical methods for separation of hulls

from RSM are inefficient and, therefore, dehulling is not a standard practice in RS extraction plants. Since the SNP in RS mainly exists in the cotyledons, this problem could not be solved through dehulling.

Other studies on the effects of processing on SNP content in RSM are very limited. Dry extrusion and other heat treatments have been unable to substantially alter the SNP content of RSM (Fenwick et al., 1986; Mansour et al., 1993). In contrast, Jensen et al. (1991) reported that heating decreased the content of SNP and this was accompanied by an increase in the content of lignan-type products in RSM. Chemical treatments offer some potential to reduce SNP content. Mustakes et al. (1968) treated crushed seed and defatted meal of *Crambe abyssinica* by cooking with sodium carbonate and steam under pressure. This treatment destroyed the glucosinolates and SNP, and improved the palatability of the meal for cattle and chickens. McGregor et al. (1983) found that gaseous ammoniation of mustard meal (*Brassica juncea*) removed up to 74% of SNP. Bell (1981) applied 0, 1 and 2% Na_2CO_3 solutions to RSM, heat-treated, and then fed the meal to growing pigs. Na_2CO_3 treatment reduced the meal content of glucosinolates and SNP by 20-40%, but reduced available lysine by 15-20%. Goh et al. (1982) observed that ammoniation of RSM at the time of desolventizing decreased the SNP and 5-vinyl-2-oxazolidinethione contents of the resulting meal. The greatest decreases in this regard occurred when RSM was sparged with ammonia in the presence of steam. Treatment of high glucosinolate *Brassica napus* meals with lime or ammonia, or by micronization, lowered egg tainting potential by reducing progoitrin, soluble tannin and SNP contents (Fenwick et al., 1984). However, the effects were not sufficient to prevent a rise in egg TMA levels above the tainting threshold. Shahidi and Naczk (1989) extracted ground RS

with 10% ammonia in methanol or 10% ammonia in methanol containing 5% water, prior to hexane extraction. The ammonia methanol extraction removed 82 and 50% of the esterified and free phenolic acids, respectively, originally present in the seed.

Recently, a promising method was described for the reduction of the glucosinolates and SNP contents of RS (Lucht, 1998). The method involves a long-term hydrothermal treatment at alkaline pH (4% Na_2CO_3) with a subsequent high temperature-short time expander treatment. Following the treatment, SNP was reduced to barely detectable levels and glucosinolates were reduced to 10% of their original value. Another experiment using the same processing method demonstrated a 40% reduction in the TMA content of egg yolks in comparison to conventional processing (Jeroch et al., 1998). Bird performance and egg quality were not affected by processing technique. However, it is hard to say that long-term hydrothermal treatment is a good way to breakdown SNP in RSM because this process may damage protein and make some amino acids unavailable. It was found that total lysine was reduced by up to 20% after high temperature or moist-heat treatment during desolventizing-toasting (Liu et al., 1993).

Meal color relates to phenolics. The dark color of RSM is not caused only by the dark or brown seed coat (hull) in the meal, which usually consists of lignin components (including phenolics), but also by heat processing during RS crushing. The contribution of physicochemical changes in these components to the palatability properties of RSM has never been evaluated.

A negative consequence of commercial RS processing is the dark-colored meal remaining after oil extraction and toasting. In current canola processing systems, the severe moist-heat treatment in the cooker and desolventizer unit darkens the meal color

and denatures protein (Liu et al., 1994; Newkirk and Classen, 2001). The resulting commercial CM is less than ideal for food or feed use partly because of the dark colour and bitter flavour associated with products produced during processing (Sosulski, 1979; Shahidi, 1992). The phenols are an important consideration in relation to the taste, colour, and nutritive value of processed RSM (Brummett et al., 1972; Maga et al., 1973). Heat and pressure seem to have significant effects on the color and structure of phenolics in RSM. Thus, the observations of a dark meal or protein isolate have been frequently reported to be associated with phenolics (Shahidi, 1992, 1995).

In summary, it seems that a combination of chemical and physical treatments, especially under alkaline conditions, is more effective than physical treatment in the removal of phenolics in RSM. However, nutrient loss during these treatments seems inevitable. Moreover, under alkaline treatments, the hydrolysis or breakdown products of phenolics in the meal and their impacts on meal protein quality and animal response are not known. In addition, the formation of quinones or their derivatives from phenolics during RSM processing requires investigation.

2.8.3 Enzymatic method

Recently, Lacki and Duvnjak (1996, 1997, 1998) proposed an enzymatic method to decrease the phenolic content in canola meal. Using an enzyme secreted by a white rot fungus, *Trametes versicolor*, the SNP content in CM was decreased by 90% (Lacki, et al., 1996). In further research, Lacki et al. (1998) proposed an enzymatic process method based on the addition of an enzyme preparation from the same white-rot fungus to concentrated meal-buffer slurries. The enzymatic process was carried out in the presence

of hexane as the main component of the continuous phase. After 1 h of treatment at 30 C, the meal simple phenolic content was decreased by 97%, but the enzyme reaction products were not reported. The products of polyphenol oxidase (oxidation) treatment are likely quinones or quinone derivatives which are very biologically active compounds and more toxic than the original phenols. Therefore, this method of enzymatic detoxification can be questioned as a viable option in the SNP reduction of RS.

2.9 Summary

Simple phenolics in RSM may affect the quality and utilization of RSM in animal diets. However, SNP and SA, which contribute the majority of total phenolics in RSM, have received little attention in animal feeding research.

Knowledge of the basic physicochemical and biological characteristics of simple phenolics in RSM is also incomplete, which may have an impact on the understanding of the nutritional properties of these compounds and the potential modification during RSM processing. Simple phenolics may have significant effects on the visual attributes, flavor characteristics and nutritional quality of RS products for animals. Nutritional or biological effects which they might have remain ambiguous. It is important to understand the role of simple phenolics in RSM in animal feeding so as to establish the need or desirability of eliminating them by plant breeding and/or processing techniques.

The reduction of levels of simple phenolics in RSM has not been successful. Physical, chemical and enzymatic treatments have been effective to some degree, but have not been adopted because of their impacts on the effectiveness and efficiency of

processing, the nutrient profile of the meal, and concerns regarding the structure and function of secondary phenolic products derived from processing.

3. QUANTITATIVE EXTRACTION AND ISOLATION, QUANTIFICATION AND PHYSICOCHEMICAL STUDIES OF SINAPINE AND SINAPIC ACID

3.1 Development of quantitative extraction, isolation and identification techniques for sinapine in rapeseed meal

3.1.1 Introduction

A laboratory procedure for the isolation of sinapine from white-mustard seed was described by Clandinin (1961). Since that time, most published research work has followed his example. However, none of the research published subsequently was able to match the SNP yields achieved in the original work by Clandinin. This is likely due to modifications of the procedure, including that of Clandinin who later applied a rotavapor instead of a water bath to concentrate the ethanol extract to a syrup. The procedure was not descriptive on how to quantify the volume of ethanol extract concentrate. The original procedure also was primarily suited for laboratory use and does not lend itself to large-scale extraction and isolation because of the time, labour and solvents required. In addition, the use of trichlorethylene (TCE) was considered a risk because it is known to be a strong carcinogen.

Large amounts of purified RSM sinapine (SNP) are required to study its nutritional effects in a broiler feeding trial. Quantitative phenolic extraction from plant material has proven to be a difficult task (Van Sumere, 1975; Shahidi, 1995) due to the complexity of the required extraction conditions and the number of factors that can affect

the process. Large-scale (pilot-scale) quantitative extraction of phenolics is yet more difficult because of the decreased control of extraction and isolation conditions. Therefore, extraction of the required SNP presented a considerable challenge. To save solvent and labor costs, and to reduce the risks of using TCE, the classical procedure was modified in the current research. In addition, the identification of purified SNP was conducted by several physicochemical techniques, including melting point and mass spectrometry.

3.1.2 Materials and Methods

3.1.2.1 Classical method

In the procedure of Clandinin (1961), expeller-processed mustard seed was used as a source of SNP. The procedure consisted of isolation of sinapine as SNP thiocyanate and conversion of SNP thiocyanate to SNP bisulfate. Since residual fat and resinous material in the meal decreased the efficiency of extraction of SNP, trichlorethylene (TCE) extraction was applied to the meal. After 4 h of TCE extraction in a soxhlet apparatus, hot 95% ethyl alcohol was applied to the extracted meal. The resulting alcoholic extract was concentrated on a steam bath to a syrup, and then diluted with water. Crystals of SNP thiocyanate were obtained by adding 20% KSCN to the extract and storing the mixture in a refrigerator for 48 h. The wet crystals were dissolved in hot alcohol and then stored for re-crystallization. A total of 4.0 g of crude SNP thiocyanate was obtained from 500 g of original meal. In a subsequent step, SNP thiocyanate was mixed with concentrated sulfuric acid several times, followed by crystallization in

distilled water and re-crystallization in alcohol to purify anhydrous SNP bisulfate. The latter compound was found to have a melting point (MP) of 187-188 °C. This procedure required approximately two weeks to extract a 500 g sample.

3.1.2.2 Modifications in the quantitative extraction and isolation of SNP from RSM

A modified method was developed to more readily match the requirements of large-scale extraction. Modifications included the elimination of the TCE extraction step and an increase in the number of times the crystallization / re-crystallization cycle was applied during the isolation of SNP (Figure 3.1, see dashed lines). The commercial RSM used in the procedure contained low levels (<5 %) of residual oil. The elimination of the TCE extraction step saved time and solvent cost, and avoided contact with this carcinogenic solvent. To compensate for any potential interference by remaining fat and resin residues on crystal formation, re-crystallization steps were repeated two more times (total of 3 times) to maximize sinapine crystal recovery. In addition, a pilot-scale ratovapor was adopted to concentrate the ethanol extract (POS Pilot Plant Corporation, 118 Veterinary Road, Saskatoon, SK).

Food-grade ethanol (95 %) was used for extraction to ensure that the SNP bisulfate crystals or concentrated SNP extract syrup was non-toxic to chickens when added to experimental diets. In the modified method, 10 kg of commercial canola meal was used as one batch for extraction and isolation. All of the solvent was applied to the process in proportion to that used by Clandinin (1961). Ideally, the meal sample should be plunged as rapidly as possible into boiling alcohol. The ethanol extract was concentrated in the rotavapor under vacuum, at neutral pH and 45 °C. For one batch, 30

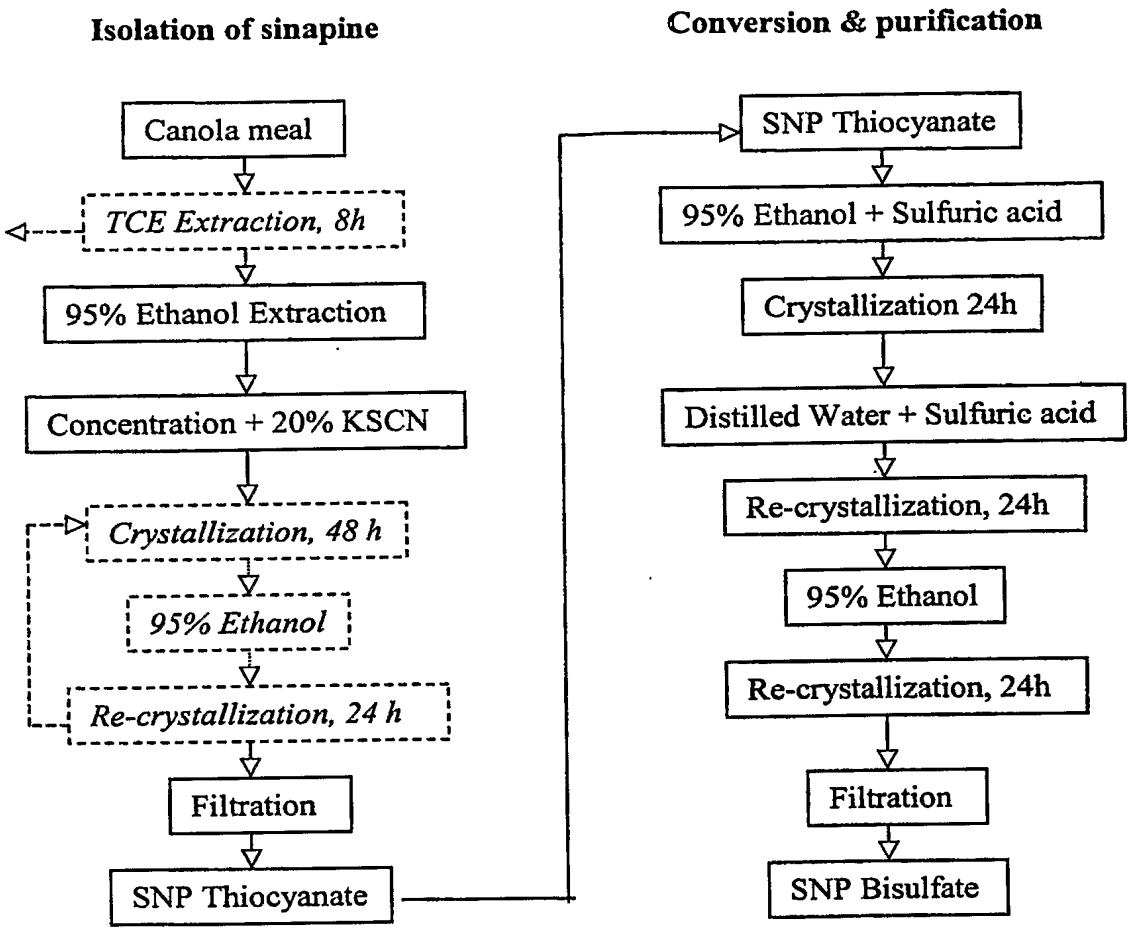


Figure 3.1 Quantitative extraction and isolation of sinapine from canola meal based on Clandinin (1961). Author modifications are indicated by dashed lines, and included the elimination of TCE extraction and an increase in the number of times the crystallization / recrystallization was applied.

L of ethanol extract was concentrated to a deep brown colored syrup with a total volume of 0.8-1.2 L, which was then diluted with distilled water and potassium thiocyanate solution was added. After the first crystallization, the extract needed to be re-crystallized at least twice.

3.1.2.3 Identification of sinapine isolates

Identification of SNP and the determination of its purity was conducted by the measurement of its melting point, Electrospray Ionization Mass Spectrometry (ESI-MS), Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS), and Collision-Induced Dissociation (CID) Tardem Mass Spectrometry (MS). The melting point was measured using a Fischer brand hot plate (Department of Chemistry, University of Saskatchewan, Saskatoon, SK Canada). Each sample was measured three times. All mass spectrometry was conducted in the Mass Spectrometry Laboratory of the Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK Canada).

ESI-MS works by applying a high voltage to a narrow capillary made of conducting material (e.g. stainless steel) through which a solution containing the sample is passed. The high electric field results in the formation of charged droplets containing a high concentration of negative or positive ions, depending on the polarity of the applied voltage. As the droplets evaporate, their charge density increases until ions are eventually released into the gas phase and can be transferred to a mass analyzer. The ESI-MS instrument uses a quadrupole mass analyzer, which has unit mass resolution (much less than with a reflectron TOF analyzer) and separates ions according to their m/z

ratios. Both components of the ionic compound, using positive ion mode for the SNP cation (m/z 310) and negative ion mode for the HSO_4-counter-anion (m/z 97), were detected. CID-MS was also used to identify the daughters of SNP cation when it was broken down into several fractions by electrical energy.

MALDI-TOF MS works by co-crystallizing the sample with a UV absorbing matrix compound (typically alpha-cyano-4 hydroxycinnamic acid) and irradiating it with a UV laser. Energy is absorbed by the matrix and transferred to the sample compound, resulting in desorption and ionization. The TOF mass analyzer separates the ionized compounds according to their mass-to-charge ratios, giving a mass spectrum of relative abundance versus m/z ratio. When operated in reflectron mode, the TOF analyzer provides very accurate mass measurements which confirm the elemental composition of the target compound. The system conditions used in the identification of purified SNP by mass spectrometry methods are listed in Tables 3.1 and 3.2.

3.1.3 Results and Discussion

It was found that SNP could be extracted and isolated from commercial canola meal using the modified procedure. The elimination of TCE extraction prior to the ethanol extraction proved to be successful. This saved the solvent and labor costs and eliminated the risk of contact with TCE. Although residual oil levels in hexane-extracted RSM are low, oil droplets were seen floating in the ethanol extract. It was found that the first crystallization was not complete, which suggests that these oil residues may interfere with the formation of SNP thiocyanate crystals. Therefore, re-crystallization must be conducted at least twice in the process to recover more SNP thiocyanate crystals.

Table 3.1 Conditions used for identification of sinapine bisulfate by Electrospray Ionization Mass Spectrometry (ESI-MS).

Tuning Parameters	ES+	ES –
Instrument (Mass Spectrometry)	Quattro LC (Micromass)	Quattro LC (Micromass)
Capillary (kVolts)	3.75	2.75
Cone (Volts)	25	25
Source Block Temp. (°C)	85	80
Neb Gas Flow (L/hr)	90	91
Desol Gas Flow (L/hr)	351	350
Scan duration (secs)	1.99	1.99
Interscan delay (secs)	0.11	0.11
Ionization mode	ES+	ES –
Function type	Scan	Scan
Mass range	2 to 502	2 to 502

Table 3.2 Conditions used for identification of sinapine by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) and Collision-Induced Dissociation Tardem Mass Spectrometry (CID MS).

MALDI-TOF Mass Spectrometry		CID Mass Spectrometry	
Instrument	Applied Biosystems	Instrument	Quattro LC (Micromass)
Mode of operation	Reflector	Tuning Parameters	ES+
Polarity	Positive	Capillary (kVolts)	3.75
Accelerating voltage (V)	20000	Cone (Volts)	25
		Source Block Temp. (°C)	85
Grid voltage (%)	72.5	Ion Energy (Volts)	0.8, 2.0
Guide wire 0 (%)	0.01	LM Resolution	16.6, 15.0
Extraction delay time (nsec)	175	HM Resolution	16.4, 15.0
		Gas Cell	9.6e-4
Number of laser shots	200/spectrum	Neb Gas Flow (L/hr)	91
		Desol Gas Flow (L/hr)	348
Laser intensity	2956	Scan duration (secs)	1.99
Calibration matrix	a-Cyano-4-hydroxycinnamic acid	Interscan delay (secs)	0.11
		Ionization mode	ES+
		Function type	Daughters of 310
		Mass range	2 to 502
		Collision Energy	29.6

After four batches, one hundred and forty grams of sinapine bisulfate was obtained and identified as the sinapine bisulfate trihydrate salt, with shiny and light yellow colored needle crystals. This sample was used for SNP analysis, the chicken feeding trial, enzymatic assays and physicochemical studies in subsequent sections and chapters.

The application of a pilot-scale rotavapor in the evaporation of the ethanol extract significantly reduced the time needed for the isolation of SNP. Concentration is a rate-limiting step, which is also a key factor in determining of the yield, since vacuum and temperature are closely related to the effectiveness and efficiency of concentration and crystallization. Neutral pH (7), vacuum, and 45°C were suitable for this procedure. However, it can be speculated that SNP yield might be higher if this process was conducted at a lower temperature (25-30°C). A lower temperature is especially necessary with labile components such as cinnamic acids, and when acids have been used for extraction (Van Sumere et al., 1972). In addition, alcoholysis is possible with some aromatic esters; therefore, care should be taken not to concentrate the solution too much. Concentration of the ethanol extract to a condensed syrup (from 30 L to 1 L) was appropriate for this step. A comparison of the modified method with the Clandinin (1961) procedure is presented in Table 3.3.

The identity of the isolated compound was initially confined by determining melting point. The crystals were found to have a decomposition point (DP) of 129-130°C, and a melting point (MP) of 188-190°C. The melting point was 1-2°C higher than the value (187-188°C) reported by Clandinin (1961) but consistent with the results of Wang (1992). The decomposition point is very close to the value given for sinapine

Table 3.3 A comparison of the modified and Clandinin (1961) procedures for the isolation of sinapine (SNP) from rapeseed meal.

Methods	Usage	Sample	Weight (kg)	Oil removal
Clandinin	Laboratory	Mustard meal	0.5	TCE
Modified	Pilot scale	Canola meal	10	None
	Concentration	Recrystal. (x)	Yield (g/kg)	Period (days)
Clandinin	Waterbath (100 °C)	3	7	10
Modified	Rotavapor (vacuum, 45 °C)	5	3.5	14

Recrystal. – recrystallization.

bisulfate trihydrate (127°C) in the Merck Index (1989).

The purity of the compound was identified by MS analysis, including Positive Ion Electrospray MS to detect sinapine, Negative Ion Electrospray MS for counter anion (bisulfate), and Collision-Induced Dissociation (CID) Tardem MS. The MS chromatogram identifications of positive and negative ions are shown in Figures 3.2 and 3.3. There were no significant peaks other than m/z 310 (sinapine) in positive-ion mode, and m/z 97 (bisulfate) in negative mode. Thus, it was concluded that the sample was essentially pure sinapine bisulfate. A further identification (confirmation) of the chemical structure of SNP by CID-MS is shown in Figure 3.4. The SNP positive ion was broken into fragments by collision energy. Measurements of the molecular weights of all fragments were matched with the chemical composition of SNP. In addition, it was found that there were traces of acetate (m/z 59) and nitrate (m/z 62) in the negative-ion spectrum, and of a compound at m/z 282 (Figure 3.5) in the positive-ion spectrum. However, these are known to be systematic impurities in the electrospray MS, and therefore were disregarded. Another smaller impurity peak was not identified but again may have been system related. The sinapine bisulfate crystals were determined to have a purity of 96.3% or higher.

Furthermore, the sample was run by MALDI-TOF MS and the exact molecular weight of sinapine (310.16) was identified, which is the correct monoisotopic mass and corresponds to the m/z ratio of the (first) isotope peak for sinapine (Figure 3.6). The MW of sinapine as 310.38 (Merck Index, 1989) is the average molecular weight, which includes all isotopes of hydrogen, carbon, nitrogen and oxygen in the molecule.

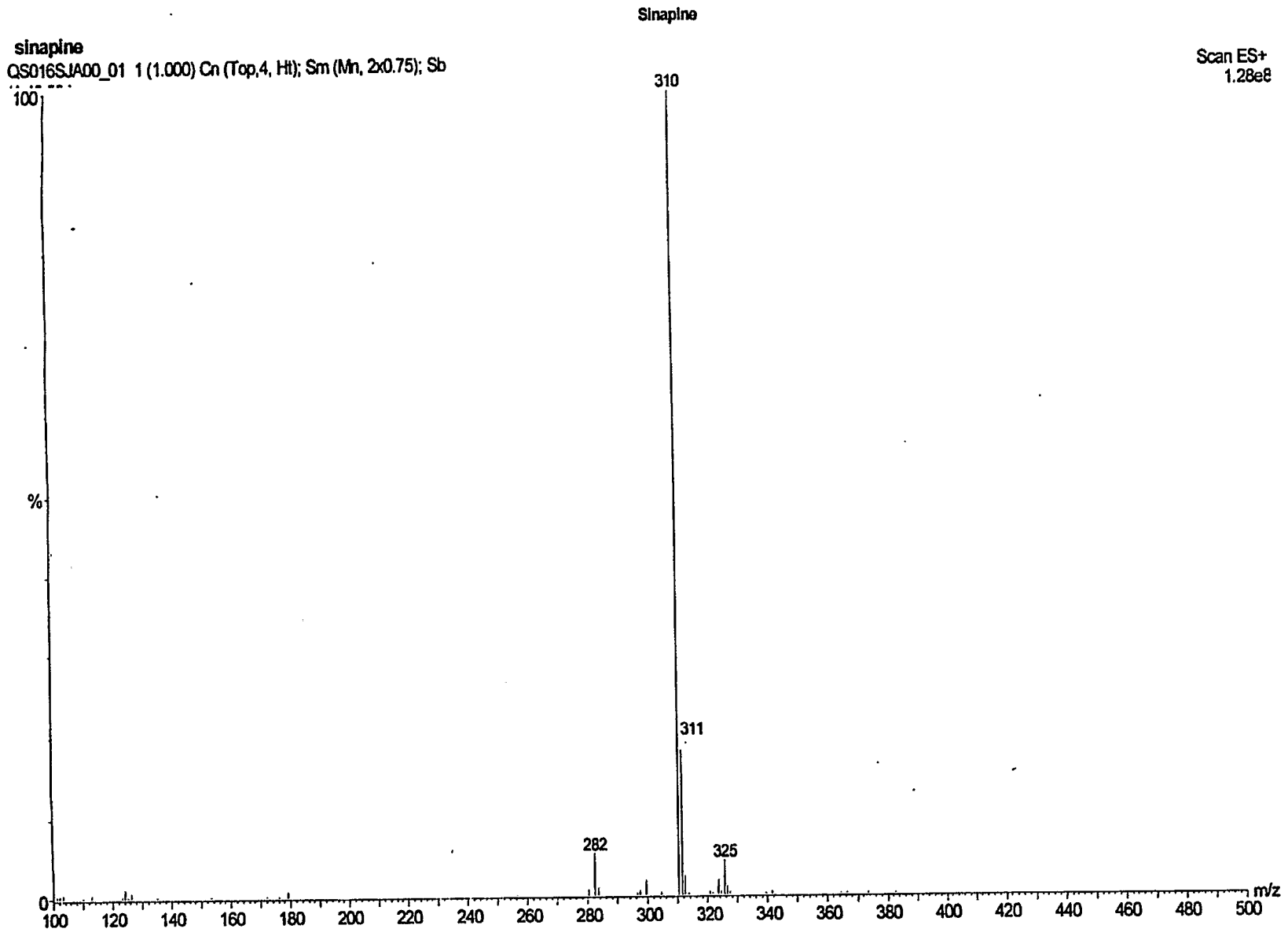


Figure 3.2 Positive Ion Electrospray Mass Spectrometry of sinapine bisulfate.

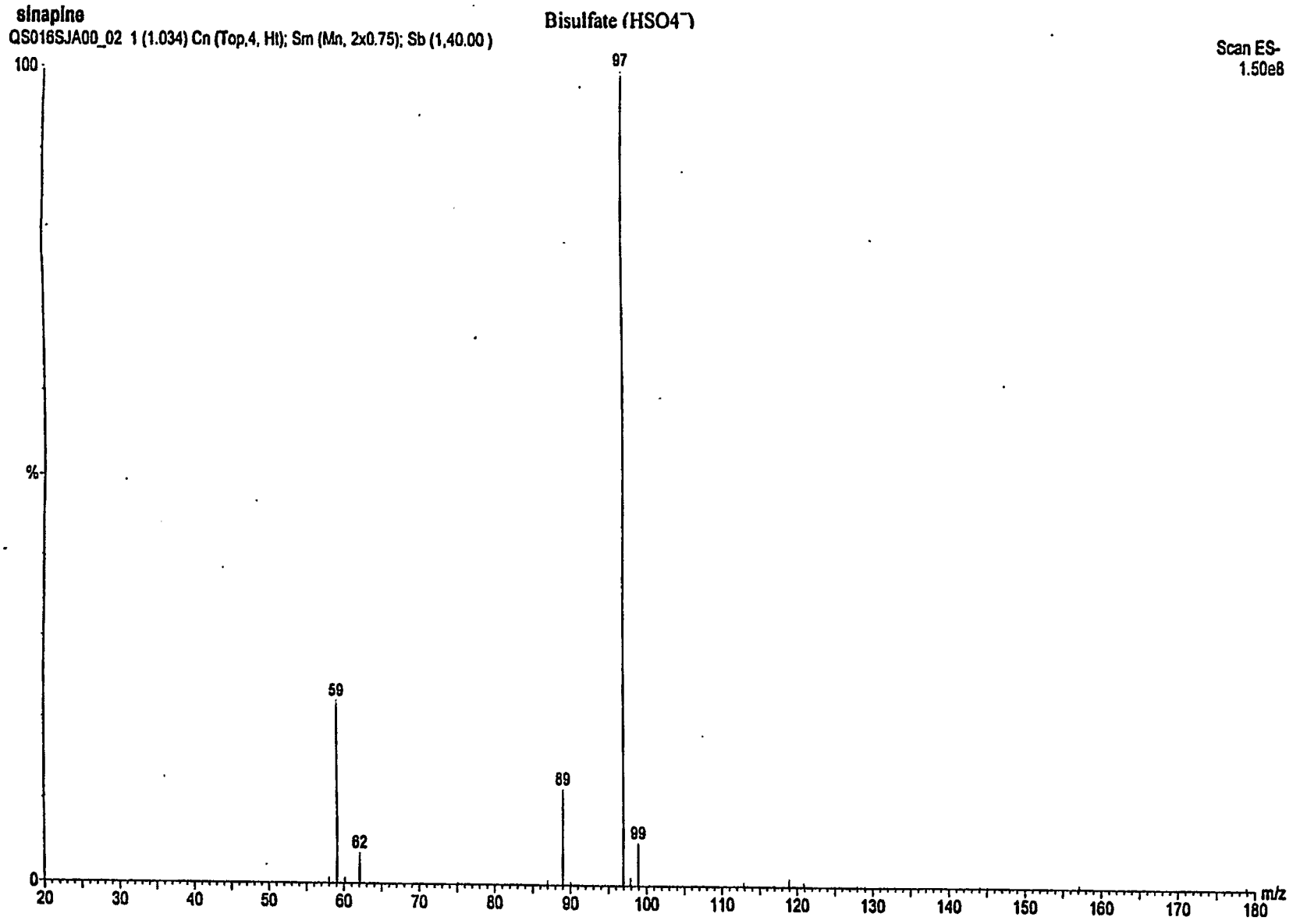


Figure 3.3 Negative Ion Electrospray Mass Spectrometry for counter anion of sinapine bisulfate.

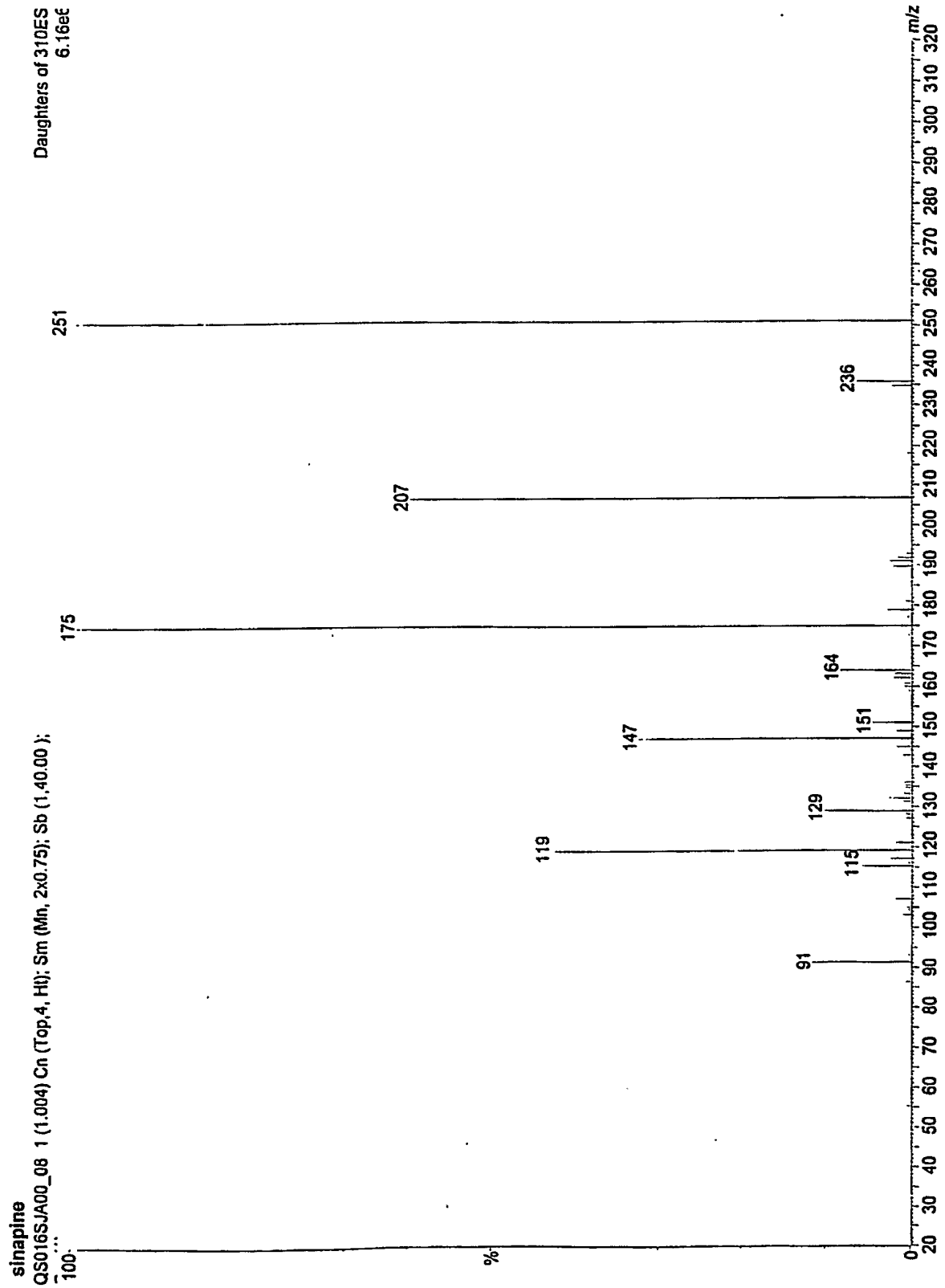


Figure 3.4 Collision-Induced Dissociation (CID) Tardem Mass Spectrometry of sinapine bisulfate.

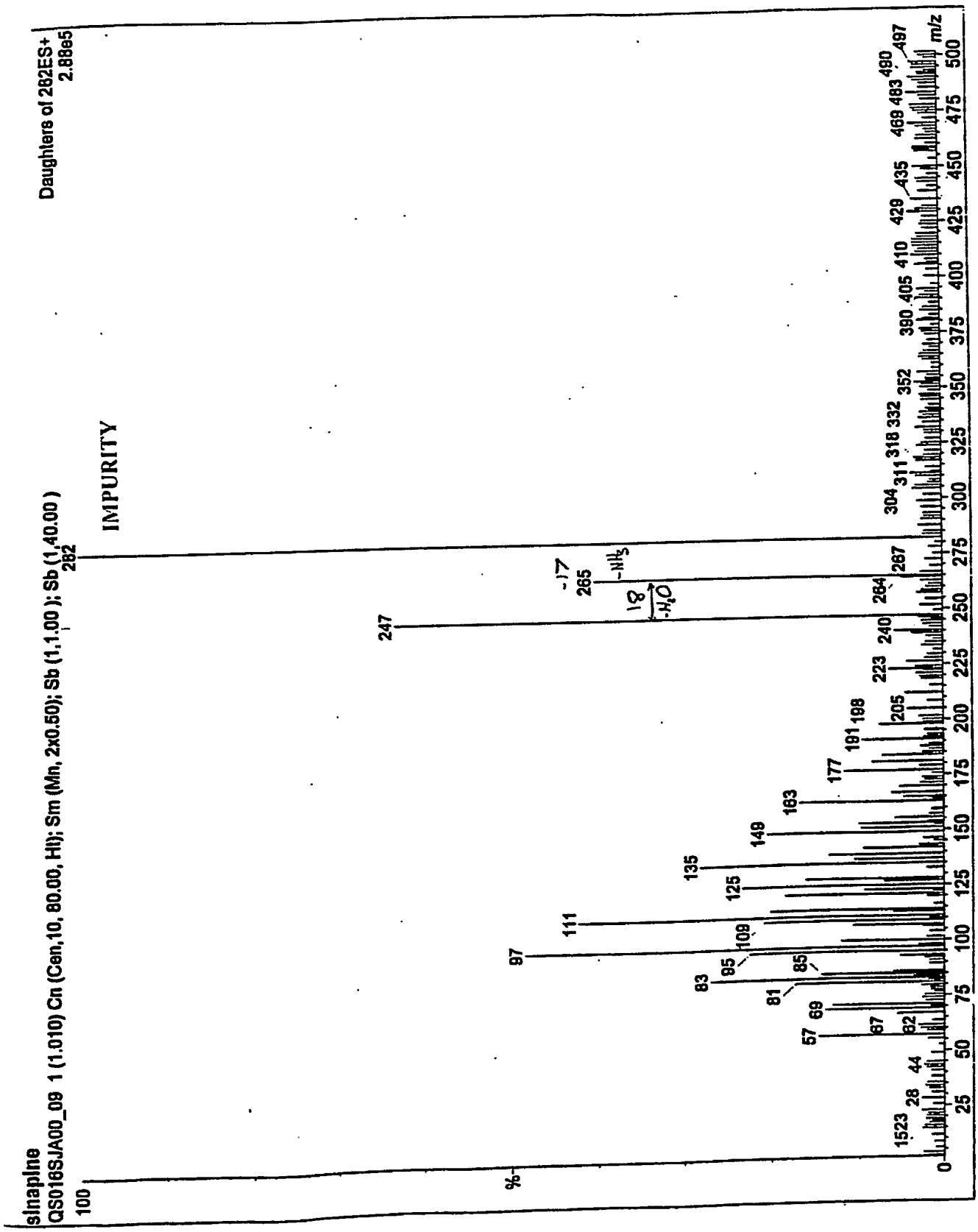


Figure 3.5 The identification of a systematic impurity in the Electrospray Mass Spectrometry of sinapine bisulfate.

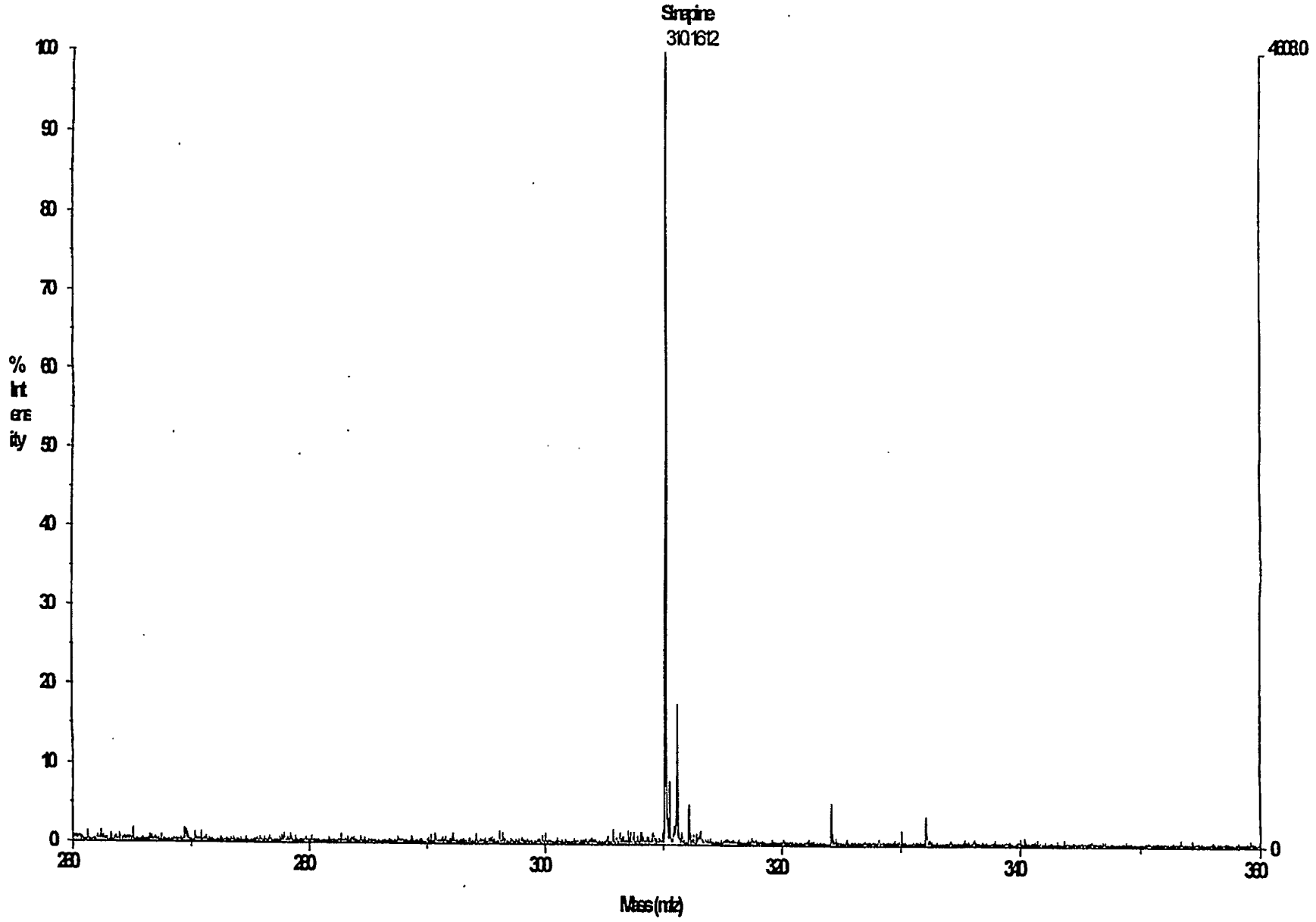


Figure 3.6 Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) Mass Spectrometry of sinapine.

3.2 Development and improvement of the quantification techniques of sinapine and sinapic acid in different biological medias

3.2.1 Introduction

For the quantification of simple phenolics in RS meal or products, most of the published work has focused on the development of sinapine assay techniques. However, some assays can be applied to measure SA as well, but these have received little attention because of the relatively low SA content of RSM. An accepted standard quantification method for SNP or SA has not been established. This is one major reason that techniques utilized to date have not given consistent results, and sometimes underestimate or overestimate the SNP content of samples.

The principles applied to SNP quantification have been based on either spectrophotometric or chromatographic methods. Spectrophotometry is the most common approach, and is based on the absorbance properties of SNP and SA. For example, the maxima wavelength for SNP and SA are 328 and 310 nm, respectively. The advantages of spectrophotometric methods include their simplicity, ease of assay and the short time required for assay. The disadvantage of the technique is its relative non-specificity (Wang, 1992; Lacki et al., 1996). A UV-spectrophotometric method with ion-exchange separation prior to assay has been proposed to enhance the accuracy of measurement (Wang, 1998).

Gas chromatography (GC) has been successfully used to determine phenolic acids in RS, sometimes together with mass spectrometry (Krygier et al., 1982; Dabrowski et al., 1984). With the development of HPLC technology, separation and reproducible

quantitative measurement of the sinapine ester is possible but a mandatory sample preparation is needed (CM-Sephadex C25 ion-exchange column) to separate SNP from other phenolic choline esters prior to analysis (Clausen, 1983, 1985; Wang, 1992; Lacki et al., 1996). HPLC is more accurate than spectrophotometric methods in determining SNP content and also provides information on the presence of other phenolic choline esters. However, HPLC is relatively time-consuming.

To study the metabolic effects of SNP and SA in chickens, an accurate analytical method was needed, since SNP and SA may be distributed in trace amount in various biological media, making them difficult to detect. To increase the sensitivity of detection using HPLC, a fluorescence detection method was applied instead of the traditional UV detector. Fluorescence methods are more precise (50 times more sensitive) and simpler than UV absorption methods. However, applications of these methods have usually been restricted to the identification of chromatographic spots and the compound to be measured must be fluorescent. There is relatively little information in the literature on the spectral properties of cinnamic acid and its derivatives.

The objectives of this research were to test the specificity of the spectrophotometric method using a scan spectrophotometer, and to develop simple and high sensitivity procedures for SNP and SA analysis in various biological media using HPLC. In addition, to facilitate the analysis of simple phenolics, an attempt was made to develop a HPLC procedure suitable for both SNP and SA and also to develop a technique specifically for SA.

3.2.2 Materials and Methods

3.2.2.1 Spectrophotometric method

SNP and SA standard solutions of the same concentration were mixed together, in the following proportions (v/v) of 100:0, 50:50, 67:33, and 33:67, and then detected with full chromatograms from 200 nm to 700 nm by using a scan spectrophotometer (Milton Roy Spectronic 3000 Array, Milton Roy Company, Serial # DEM017, 2001 Ivyland Road, Ivyland, PA 18974 USA).

3.2.2.2 HPLC method

Sample preparation. Feed or meal samples (50 mg) were ground using a 0.5 mm screen and added to a 15 mL screw-top test tube containing 5 mL of 70 % methanol (acidified with 1% hydrochloric acid). The contents of the tube were mixed, the tube capped and heated at 75°C in a waterbath for 20 min with mild shaking. Another 5 mL of 70% acidified methanol was added to the cooled extract and the extract centrifuged at 2500 rpm for 10 min. The supernatants were transferred to HPLC vials for SNP or SA analysis. The weights used for other samples were as follows: feces, 50 mg; ileal samples, 0.15 mg; and cecal samples (wet), 0.50 mg. All samples were mixed well before sampling.

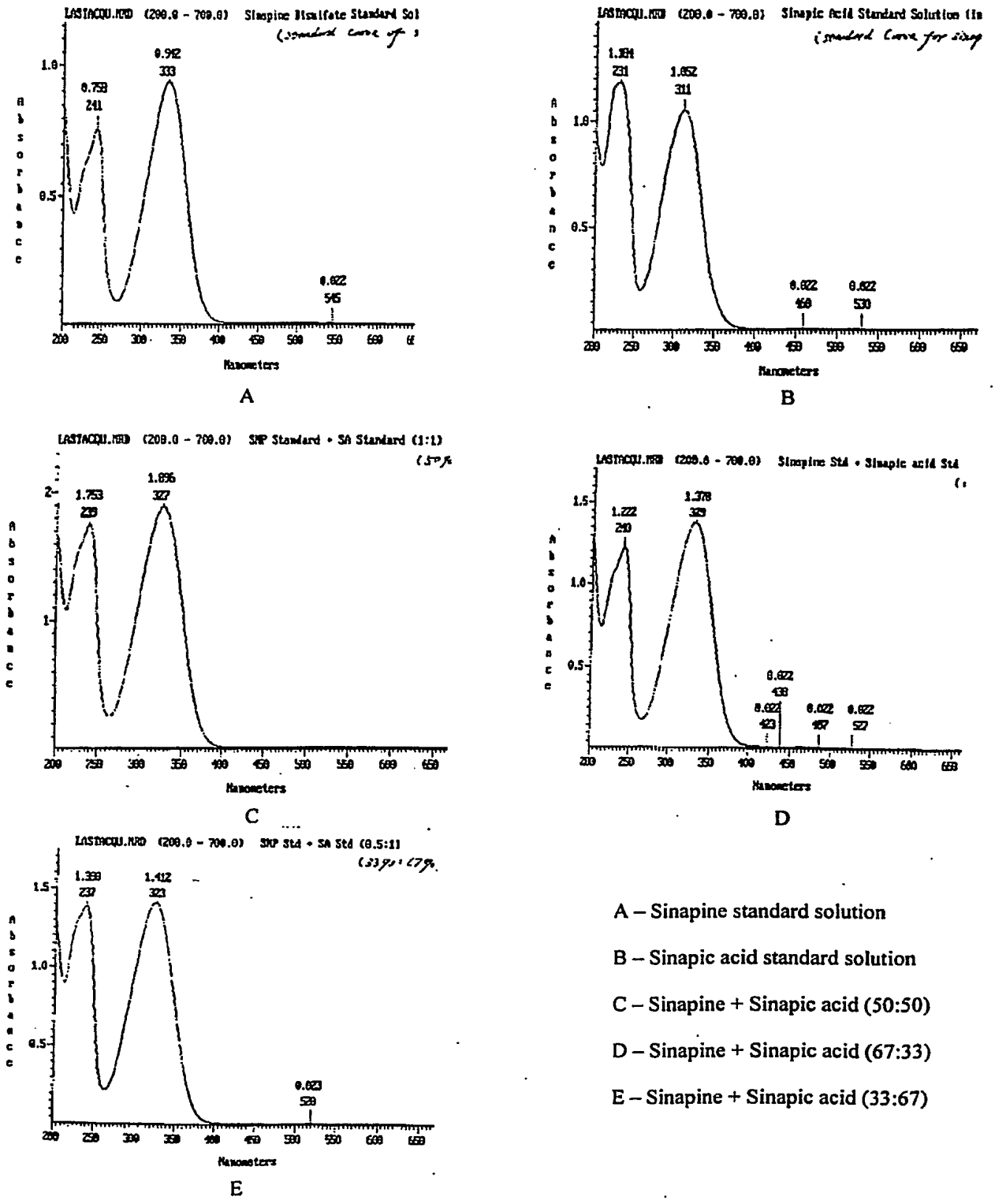
Detection method, HPLC column and mobile phase selection. SNP and SA were detected and the excitation and emission wavelengths for both compounds were measured using a fluorescence detector (Shimadzu RF 551 spectroflurometric, Serial # 30077K, Shimadazu Scientific Instruments, Inc. 7102 Riverwood Drive, Columbia,

Maryland 21046 USA). The analysis of SNP and SA was conducted by HPLC (Beckman System Gold, Beckman Instruments, Inc., Palo Alto, CA USA) with several reversed-phase columns (C_{18}, C_8) and a Hamilton polymer based column (PRP-1, 150 x 4.1 mm I.D. 5 μm, Serial # 8572, Hamilton Company, P. O. Box 10030 Reno, Nevada 89520-0012 USA), using mobile phases based on methanol or acetonitrile solutions with an acid-base modifier (potassium phosphate, monobasic- or dibasic form) for adjusting the pH.

3.2.3 Results and Discussion

3.2.3.1 Spectrophotometry

As shown in Figure 3.7, none of the chromatograms could distinguish SA from SNP, as the two peaks combined with changes of peak shape and height with different proportions of SNP and SA. SNP and SA were detected at 333 and 311 nm, respectively, for maximum absorbance; however, after mixing, the maximal absorbance wavelength shifted to 327, 329 and 323 nm for mixtures of SNP and SA at proportions (v/v) of 50:50, 67:33, and 33:67, respectively. The shift in maximal absorbance wavelength is logical since the maxima wavelength varied with the relative proportion of SNP and SA in the mixture. These results demonstrated that SNP or SA could not be accurately separated and detected by this method. This is due to the non-specificity of this method, and the fact that the physicochemical properties of SNP and SA are too similar. In RSM samples, SNP and SA co-exist and the maxima wavelength difference is less than 20 nm; thus, it is very hard to distinguish them from each other. Although the



A – Sinapine standard solution

B – Sinapic acid standard solution

C – Sinapine + Sinapic acid (50:50)

D – Sinapine + Sinapic acid (67:33)

E – Sinapine + Sinapic acid (33:67)

Figure 3.7 Spectrophotometric analysis of sinapine and sinapic acid standard solutions using a scan spectrophotometer.

spectrophotometric method may suffice for the approximate estimation of total simple phenolics in RSM, it was not appropriate for precise analysis of each compound.

3.2.3.2 HPLC chromatography

SNP and SA were detected using a fluorescence detector. The excitation (EX) and emission (EM) wavelengths of SNP and SA were first identified as: EX: SNP–318 nm, SA–280 nm; EM: SNP–465 nm, SA–428 nm. A reversed-phase polymer based Hamilton PRP-1 column was employed. The mobile phase used was a methanol solution with potassium phosphate as the buffer system. SA and SNP were detected at the same wavelengths.

Specific mobile phases applied to assay SNP and SA are summarized in Table 3.4. For SNP analysis, a 6 min elution time was needed to analyze one sample. Because of the presence of other compounds with elution times close to that of SA, a total assay time of 22-24 min was necessary to separate these compounds from SA. Typical chromatograms of SNP and SA in different samples are shown in Figures 3.8 and 3.9.

Attempts to simplify the analysis of SNP and SA by using the same mobile phase were unsuccessful because of their different spectral properties under specific system conditions. In addition, the mobile phase needed to be modified for specific samples due to the complexity of the medium and the presence of other compounds in the sample capable of interfering with the analysis. However, an analysis based on the excitation and emission detection wavelength which is optimal for SNP using fluorescence detection, and the use of an isocratic mobile phase, has been successful in measuring both SNP and SA, even without removing other phenolic choline esters by CM-Sephadex C25

Table 3.4 Mobile phases and elution times of SNP and SA for a reversed-phase column using HPLC for quantification in different biological media.

Media	Mobile phase	Elution time
Sinapine		
Feed, feces and ileal samples	35 % MeOH, 20 mM KH_2PO_4 (pH 4.0)	3.9-4.0 m
Cecal samples	28 % MeOH, 20 mM KH_2PO_4 (pH 4.0)	N/A
Sinapic acid		
Feed and feces samples	6 % MeOH, 20 mM K_2HPO_4 (pH 9.5)	6.6-6.7 m
Ileal samples	6 % MeOH, 20 mM K_2HPO_4 (pH 9.0)	8.6-8.9 m
Cecal samples	6 % MeOH, 20 mM K_2HPO_4 (pH 9.0)	N/A

m – minute;

N/A – not applicable.

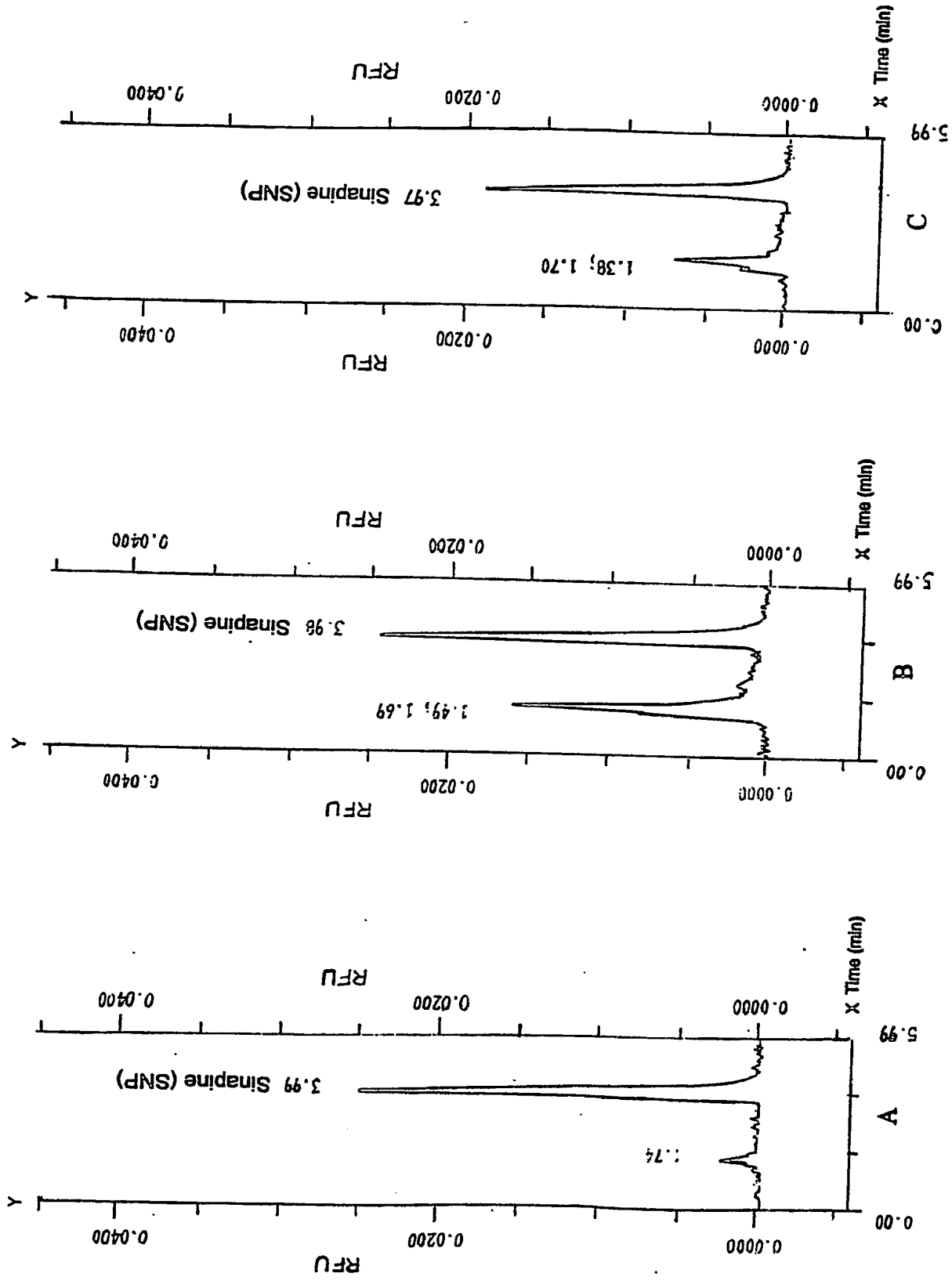


Figure 3.8 Typical chromatograms of sinapine in different biological media (A: feed; B: feces; C: ileum) by HPLC using fluorescence detection (X axis – elution time, Y axis – RFU, relative fluorescence unit).

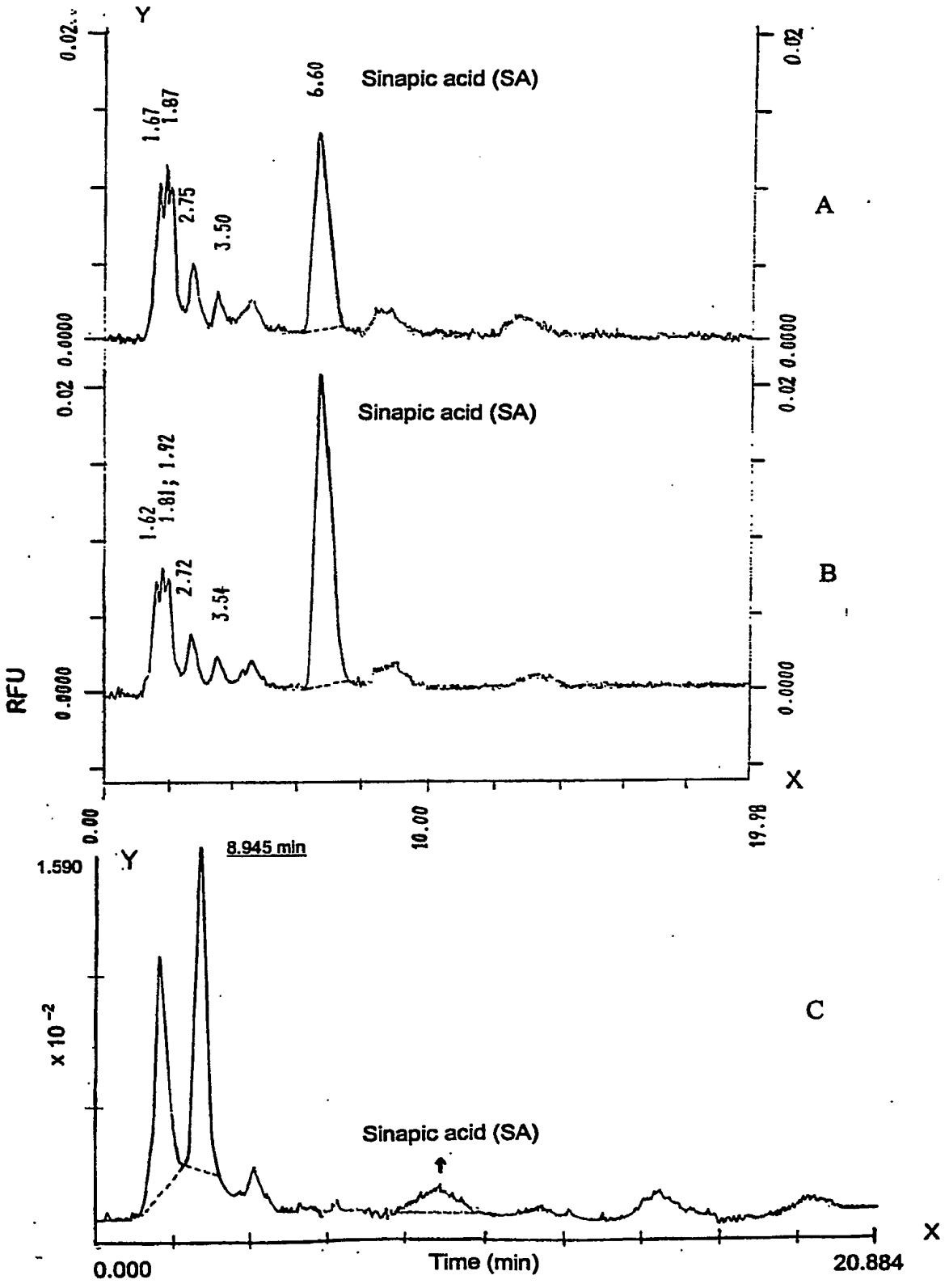


Figure 3.9 Typical chromatograms of sinapic acid in different biological media (A: feed; B: feces; C: ileum) by HPLC using fluorescence detection (X axis – elution time, Y axis – RFU, relative fluorescence unit).

ion-exchange column prior to HPLC assay.

3.3 Physicochemical studies of sinapine and sinapic acid

3.3.1 Introduction

The physical and chemical characteristics of SNP (or salt form) and SA are not completely known. However, an understanding of the physicochemical characteristics of these compounds and accurate quantification techniques are key factors in disclosing their biological effects in animals. Some characteristics may be closely related to their function in nutrient digestion and absorption, and their metabolism within the body of an animal. Some characteristics of these compounds have been noted in current research during SNP isolation, the development of quantification techniques, and enzymatic assays, but our understanding may still lag behind the real impact these compounds might have on animals, either directly or indirectly.

A fluorescence detection technique was introduced in the HPLC analysis to enhance the sensitivity of detection and simplify the HPLC procedure. Due to the presence of small amounts of ferulic acid, *p*-coumaric acid, and caffeic acid in RSM, which have similar chemical structures to SA, it is also essential to separate them from SA during chromatography.

Based on preliminary research, it was hypothesized that the spectral properties of SNP bisulfate were not the same under different pH conditions.

3.3.2 Materials and Methods

3.3.2.1 Physicochemical property measurements

Solubility. SNP bisulfate trihydrate and sinapic acid were tested for solubility in several solvents, namely, deionized water, ethanol, methanol, acetone and acetonenitrile.

Decomposition and melting points. SNP thiocyanate and SNP bisulfate trihydrate were measured by a Fisher brand hot plate. Each sample was measured three times.

Fluorescence excitation and emission wavelengths. The EX and EM wavelengths of SNP and SA were measured by a fluorescence detector. See section 3.2.2.2 for details.

3.3.2.2 Separation of SA from other phenolic acids

A mixture of several standard compounds, *p*-coumaric acid, ferulic acid, caffeic acid and SA, was dissolved in pure methanol with the same concentration for each compound and then measured by HPLC using both UV and fluorescence detection methods. See section 3.2.2.2 for details.

3.3.2.3 Spectral properties of SNP bisulfate

A sample (15 mg $SNP{\cdot}HSO_4{\cdot}3H_2O$) was dissolved in 10 mL of redistilled water (natural pH 5.6-5.7) or pure methanol at room temperature. One mL of each stock solution was transferred to each of 10 tubes (125x16 cm) and then 9 mL of redistilled water or pure methanol was added to make a total volume of 10 mL. In theory, the SNP

content in each tube was equivalent to 100 μg/mL. Subsequently, the pH was adjusted from 1 to 10 by adding concentrated hydrochloric acid or potassium hydroxide and then vortexing the tubes. The visual color of each tube was recorded. In addition, the two sets of samples were transferred to HPLC vials for analysis using the fluorescence detection method.

3.3.3 Results and Discussion

3.3.3.1 Physicochemical property measurements

Solubility. SNP bisulfate was very soluble in water, ethanol, methanol, acetone and acetonenitrile, whereas SA was soluble in methanol, ethanol and acetone, but not in water. This was probably due to SNP having hydrophilic properties owing to the salt structure in the choline moiety and lipophilic properties derived from the aromatic parts of the molecule. However, SA has only the lipophilic aromatic chemical structure.

Decomposition and melting point. When the SNP bisulfate crystals were heated to 129-130°C, crystallized water was seen be released as the crystals became anhydrous and opaque. At 188-190°C, the crystals melted obviously until they were all in liquid form. Thus, the isolated SNP bisulfate salt was identified as having a decomposition point of 129-130°C, and a melting point of 188-190°C (see section 3.1.3). During the isolation of SNP, SNP was extracted from RSM and precipitated as SNP thiocyanate and then converted into SNP bisulfate. The melting point of SNP thiocyanate has not been previously reported. The SNP thiocyanate salt was

determined to have a melting point of 168°C, which was lower than that of the bisulfate form.

Fluorescence excitation and emission wavelengths. The excitation and emission wavelengths of SNP and SA were first measured as: EX wavelength, SNP–318 nm, SA–280 nm; EM wavelength, SNP–465 nm, SA–428 nm, using the fluorescence detection method. The 38 nm difference for EX or EM wavelength is possibly caused by the different chemical structure of the side chains attached to the phenol group.

3.3.3.2 Separation of SA with other phenolic acids

Chromatograms from HPLC analysis using UV and fluorescence detection demonstrated that the sensitivities and elution times for each compound were similar. The elution order starting from the earliest was caffeic acid, *p*-coumaric acid, ferulic acid and SA (Figure 3.10). Moreover, it was found that this order remained the same with changes in the methanol based mobile phase within each method.

3.3.3.3 Spectral properties of SNP bisulfate

As shown in Table 3.5, a colored substance was formed with increasing pH. In redistilled water, from pH 7 to 10, the color changed from very light yellow to yellow, whereas in methanol, from pH 8 to 10 the solution was light yellow in color with no visual difference in this pH range. At the same time as the pH and color changed, the detectable SNP content decreased. In comparison to the expected level, the SNP content was 24 and 18% less at pH 1, and 40 and 60% less at pH 10, in water and methanol, respectively. This study demonstrates that the spectral properties of SNP bisulfate

Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1	Caffeic	1.795	101293	6549		MM
2	Cumaric	3.312	170409	8897		MM
3	Ferulic	7.312	187586	5723		MM
4	Sinapic	11.212	71319	1703		MM

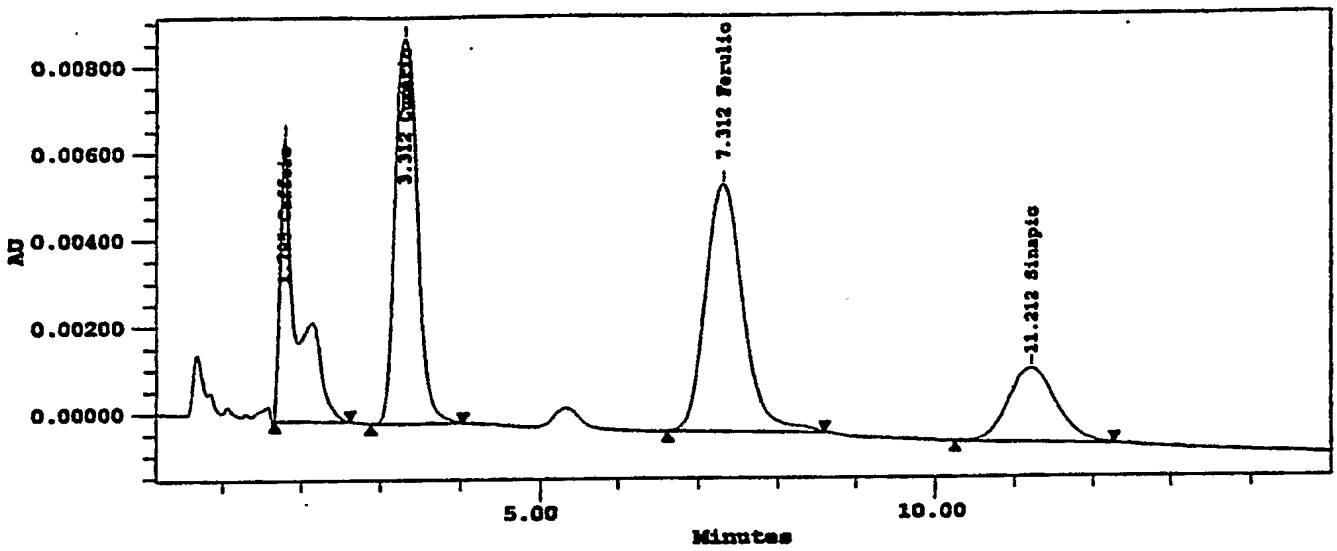


Figure 3.10 The appearance and elution order of caffeic, *p*-coumaric, ferulic, and sinapic acid on a reversed phase column by HPLC using UV absorption detection.

Table 3.5 Physicochemical changes of sinapine bisulfate in redistilled water or methanol under different pH conditions at room temperature.

Water	pH value	Concentration µg/ml	Reduction by %	Color change [1]	*Methanol*	pH value	Concentration µg/ml	Reduction by %	Color change
Sam. ID					Sam. ID				
No. 0		100.0			No. 0		100.0		
No. 1	1	75.8	24.2	CL	No. 1	1	81.7	18.3	CL
No. 2	2	74.6	25.4	CL	No. 2	2	84.2	15.8	CL
No. 3	3	67.5	32.5	CL	No. 3	3	74.1	25.9	CL
No. 4	4	63.7	36.3	CL	No. 4	4	73.5	26.5	CL
No. 5	5	52.0	48.0	CL	No. 5	5	74.3	25.7	CL
No. 6	6	58.7	41.3	CL [2]	No. 6	6	77.2	22.8	CL
No. 7	7	57.6	42.4	VLY	No. 7	7	71.0	29.0	CL
No. 8	8	53.2	46.8	LY	No. 8	8	69.3	30.7	LY
No. 9	9	51.7	48.3	LY	No. 9	9	63.7	36.3	LY
No. 10	10	40.7	59.3	Y	No. 10	10	<60	>40	LY

[1] CL – colorless; VLY – very light yellow color; LY – light yellow color; Y – yellow color. [2] Boiled at 95-100 °C for several minutes, changed to light yellow color.

change with pH. This finding may be of considerable importance in measuring SNP regardless of the analytical method. It can be inferred that all current analysis methods may underestimate the SNP content by at least 20%. Hence, quantitative studies of SNP in buffer solutions should take into account solution pH.

Following this study, SNP bisulfate solution (pH 6 in redistilled water) was boiled at 95-100°C for several minutes. The color of the solution changed from colorless to light yellow. This suggested that the formation of a colored substance (s) was related to a structural change of SNP, either by hydrolysis under current conditions or by oxidation as a result of high temperatures. In addition, the addition of HCl into the yellow colored solutions resulted in the recovery of colorless solutions. This implies that the reaction could be reversed by adjusting pH.

The products produced under different pH conditions were not determined in this research. Therefore, more work is needed to follow up on these findings. This point may be very important in commercial RSM processing, where SNP is exposed to variable pH, temperature, moisture, heat and pressure conditions. Whether these simple phenolics would have been partially transformed during processing, or are sensitive to environmental conditions during normal handling, the quality of the meal should be re-evaluated. In addition, the products of these reactions may be anti-nutritional factors in RSM which have never been realized and which may be of considerable interest in animal nutritional studies.

4. NUTRITIONAL, PHYSIOLOGICAL, AND METABOLIC EFFECTS OF RAPESEED MEAL SINAPINE IN BROILER CHICKENS

4.1 Abstract

Research was completed to investigate the impact of dietary rapeseed meal (RSM) sinapine (SNP) on broiler chickens. The study used 10 treatments with 4 replications per treatment and 3 chicks (*Peterson* X *Hubbard*) per replication. The control diet was based on a corn-soybean meal, as were six additional treatments which contained three levels of SNP bisulfate trihydrate or semi-purified SNP in the form of an ethanol extract concentrate. The last three treatments were corn based but soybean meal was replaced by graded levels of RSM at 15, 22.5, and 30 % of the diet and thereby added SNP in its native form. The three sources of SNP provided three equivalent levels of dietary SNP (0.150, 0.225, 0.300 %). Birds were monitored from 0 to 18 days. Data collection included bird performance, relative internal organ weights and intestinal measurements and digestibility determined in ileal and fecal samples.

SNP bisulfate and SNP ethanol extract did not affect feed intake and performance of broiler chickens. Both SNP bisulfate and ethanol extract SNP increased diet AME with a quadratic response ($P<0.01$) while ethanol extract SNP also increased fecal protein digestibility ($P<0.05$) in comparison to the control diet.

Dietary treatment caused minor effects on tissue measurements. All the diets containing SNP reduced the empty weight of ceca ($P<0.05$) suggesting that the hind gut might be an important metabolic site for SNP. Diets containing RSM caused an increase

in relative liver weight ($P<0.05$). RSM treatments also increased plasma T3 and T4 levels.

The ileal digestibility of SNP was 35-42, 27-38, and 30-46 %, for SNP bisulfate, SNP ethanol extract, and RSM SNP, respectively, while fecal digestibility values were 68-72, 65-75, and 54-63 % for the same treatment groups. A major metabolic pathway of SNP via hydrolysis into sinapic acid and choline in the digestive tract was identified.

(*Key words*: sinapine, sinapic acid, rapeseed meal, digestibility, metabolism)

4.2 Introduction

Sinapine (SNP) is a choline ester of sinapic acid and accounts for 80 % of the total phenolic acids in rapeseed meal (RSM), with free SA and insoluble bound phenolics making up the remainder (Shahidi and Naczk, 1992). The SNP content in RSM has been shown to range from 0.6 % to 3.0 % depending on cultivar, and growing condition and location (Krygier et al., 1982, Wang, 1992; Shahidi, 1995; Lacki et al., 1996).

SNP is considered to possess a number of anti-nutritional effects in regard to the use of RSM in feed for non-ruminant animals. It is thought to contribute to the dark color, bitter and sour taste, and astringency of RSM (Clandinin, 1961; Maga et al., 1973; Sosulski, 1979) and the latter characteristics have been implicated in the decreased palatability of RSM in rat and pig diets (Josefsson et al., 1976; Sosulski, 1977). Research has clearly demonstrated that SNP is linked to fishy taint in eggs from some brown-egg laying hens fed RSM (Hobson-Frohock et al., 1973; Butler et al., 1982; Fenwick et al., 1984). SNP is hydrolyzed to SA and choline by intestinal bacteria and the choline is further modified to trimethylamine (TMA) prior to absorption (Oh et al., 1986). Usually the TMA is oxidized and excreted via the kidney but some hens lack or have low levels of TMA oxidase and as a consequence TMA is deposited in developing eggs and causes fishy taint.

SNP anti-nutritional effects may also be related to sinapic acid which is also released upon microbial hydrolysis in the gastrointestinal tract. Sinapic acid (SA) can bind to bovine serum albumin *in vitro*, suggesting that these simple phenolics have the potential to decrease the value of RSM as a protein source (Shahidi et al., 1995).

Oxidized phenolic compounds can bind to essential amino acids such as the ε--NH_2 group of lysine and the CH_3S group of methionine, forming complexes which are unassimilable in the digestive tract of animals and man (Davies et al., 1968; Van Sumere et al., 1975; Rutkowski et al., 1977).

With the exception of fish egg taint, the negative effects of simple phenolics have rarely been demonstrated in animal trials. This is particularly true for the use of purified SNP where only two short term experiments have been completed with rats and mice being the target species (Austin et al., 1968; Josefsson et al., 1976). Moreover, the impact of SNP on the metabolism and physiology of non-ruminant and ruminant animals has not been investigated. Despite the relatively scarcity of knowledge of the nutritional consequence of SNP, there has been considerable effort devoted to its elimination or reduction in RS. Therefore, research is required to clarify the role of RSM SNP in animal feeding.

The objectives of this experiment were to investigate the effects of dietary sinapine on broiler performance, physiological parameters, and nutrient retention, and investigate the metabolism of SNP in the chicken digestive tract. Dietary SNP was fed as a purified compound (SNP bisulfate trihydrate), in a semi-purified form in an ethanol extract concentrate, or in its native form in RSM.

4.3 Materials and Methods

4.3.1 Animals and diets

The experiment was based on a complete randomized design in which 120 male day old chicks (*Peterson* X *Hubbard*) were randomly assigned to ten treatments, with four replications per treatment and three birds per replication. The first seven treatments were based on a corn-soybean diet with one as a control, three with added graded levels of purified sinapine bisulfate trihydrate, and three treatments supplemented with increasing levels of semi-purified SNP in the form of an ethanol extract concentrate. The last three treatments contained 15.0, 22.5 and 30.0 % RSM where SNP was present in its native ester form in the meal. The three dietary levels of each SNP form were 0.150, 0.225 and 0.300 % (as % of SNP). The inclusion levels were based on the measurement of SNP content in the commercial RSM as 1.0 % prior to diet formulation.

Chicks were housed in battery brooders with room temperature automatically regulated (computer control) to meet their brooding requirements. Light was provided for 23 h per day until five days of age when it was changed to 16 h of light. Feed and water were provided *ad libitum*.

All the diets were formulated to be isoenergetic and isonitrogenous, and either met or exceeded the nutrient requirements of broiler chickens (NRC, 1994). SNP bisulfate trihydrate with a purity >96.3 % was produced from commercial RSM based on the procedure of Clandinin (1961) with modifications. See Chapter 3.1 for more details. Diets containing the ethanol extract (see Chapter 3.1) were left in the air for 48 hours to completely evaporate the ethanol residue. The composition and nutrient content of diets are shown in Tables 4.1 and 4.2.

Table 4.1 Diet composition and treatment design.

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Canola meal		
Ingredient (%) SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %
Corn	56.7	56.5	56.4	56.3	56.6	56.5	56.4	50.2	46.9	43.6
Soybean meal	34.5	34.5	34.5	34.5	34.5	34.5	34.5	24.4	19.4	14.3
SNP bisulfate trihydrate	-	0.23	0.34	0.45	-	-	-	-	-	-
Ethanol extract (g)	-	-	-	-	450	675	900	-	-	-
Canola meal	-	-	-	-	-	-	-	15.0	22.5	30.0
Canola oil	2.7	2.7	2.7	2.7	2.7	2.7	2.7	4.63	5.59	6.56
Dicalcium phosphate	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.47	1.43	1.39
Limestone	1.74	1.74	1.74	1.74	1.74	1.74	1.74	1.58	1.50	1.42
NaCl	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.43	0.42	0.41
Vitamin/Mineral premix[1]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
DL - Methionine	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.21	0.19	0.18
L - Lysine	-	-	-	-	-	-	-	0.01	0.02	0.02
Celite	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50

[1] Vitamins and minerals supplied per kilogram of diet: vitamin A (retinyl acatate + retinyl palmitate), 11000 IU; D_3, 2200 IU; E (dl-α-topheryl acetate), 300 IU; menadione, 2.0 mg; thiamine, 1.5 mg; riboflavin, 6.0 mg; niacin, 60 mg; pyridoxine, 4 mg; B_{12}, 0.02 mg; pantothenic acid, 10.0 mg; folic acid, 0.6 mg; and biotin, 0.15 mg; ethoxyquin, 0.625 mg; calcium carbonate, 500 mg; iron, 80 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; and selenium, 0.3 mg.

Table 4.2 Major nutrient profile, amino acid level and SNP content in practical diets.

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Canola meal		
Nutrient (%) SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %
AME Mcal/Kg	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
Crude protein [1]	24.4	23.3	23.6	23.6	24.2	23.9	23.2	23.7	23.5	23.2
Ca	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Non-Phytate P	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Na	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Linoleic acid	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.96	2.06	2.15
Arg	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.33	1.30	1.27
Lys	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12
Met	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
Met+Cys	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.95	0.98	1.00
Thr	0.81	0.81	0.81	0.81	0.81	0.81	0.81	0.83	0.85	0.86
Trp	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.25	0.25	0.25
SNP content [1]	0	0.124	0.177	0.249	0.094	0.129	0.158	0.113	0.172	0.219
SA content [1]	0	0	0	0	0.045	0.049	0.058	0.048	0.051	0.053

[1] Analyzed values for crude protein, SNP, and SA content.

4.3.2 Data collection

Broiler performance was monitored from 0 to 18 d with body weight and feed consumption assessed at 7, 14 and 18 d of age. Feces were collected from 16 to 18 d of age and pooled for the determination of diet AME, and protein and SNP digestibility. At the end of 18 d, the birds were blood sampled to provide plasma for triiodothyronine (T3) and thyroxine (T4) analysis. The birds were then killed by cervical dislocation, internal organs (bursa, heart, kidney, liver, spleen, pancreas, proventriculus, gizzard) and the digestive tract were removed and measured. Two sets of ileal and cecal contents were collected and pooled for the estimation of ileal digestibility and SNP and SA content.

Apparent digestibility. Apparent digestibility calculations (AME, protein, SNP) were based on the use of acid insoluble ash (AIA, Celite) as an indigestible marker. AIA was determined using the procedure of Vogtmann et al. (1975). Gross energy was measured by a traditional bomb calorimeter (AOAC, 1990). The crude protein content was analyzed by a Leco FP-528 protein analyzer (Model No. 601-500-100, Serial # 3211, LECO Corporation, 3000 Lakeview Avenue, St. Joseph MI 49085-2396 USA). Based on the nutrient and AIA content in feed, feces, and ileum, the nutrient digestibility was determined.

Triiodothyronine (T3) and thyroxine (T4) assay. T3 and T4 levels in plasma were determined by radioimmunoassay using a total T3 and T4 Kit (Sigma Chemical Co., P.O. Box 14508 St. Louis, MO 63178 USA) and a APEX^{TM} automatic gamma counter. The principle of the total T3 procedure was based on a solid-phase radioimmunoassay, wherein ^{125}I-labeled T3 competes for a fixed time with T3 in the sample for antibody sites. Total T4 procedure is based on antibody-coated tubes and human serum

calibrators. ^{125}I-labeled T4 competes for a fixed time with T4 in the samples for antibody sites, in the presence of blocking agents for thyroid hormone-binding proteins.

SNP and SA assay. The determination of SNP and SA in feed, ethanol extract, feces, and ileal and cecal digesta was based on HPLC technique using a reversed-phase column under the fluorescence detection method as previously described (Chapter 3.2).

4.4 Statistical Analysis

Internal organ and intestinal weights and lengths were expressed as a proportion of bird weight (value /body weight). Data were subjected to one-way Analysis of Variance (ANOVA) using the General Linear Model (GLM) procedure with a priori contrasts and regression analysis (SAS® Institute, 1999). Differences were considered significant when $P<0.05$, unless otherwise stated.

4.5 Results

Dietary treatment did not affect broiler weight gain and feed consumption, except for gain to feed ratio of broilers raised to 18 d of age (Table 4.3). Statistical interpretation of these data using ANOVA and a priori contrast, and regression analyses are shown in Tables 4.4 and 4.5, respectively. GLM analysis of feed to gain ratio indicated a significant treatment effect but a priori contrasts and regression analyses failed to reveal significant differences between SNP treatments and the control. The inclusion of RSM in treatment diets resulted in a numeric decrease in growth rate (linear regression, $P = 0.10$) in comparison to the control treatment.

Table 4.3 The effect of SNP source and level on the performance and relative internal organ weights of broiler chickens.

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Canola meal			SEM
SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	
Gain (g)	491	499	463	496	491	481	473	444	443	447	7.6
Feed (g)	655	668	636	659	653	618	623	599	601	618	9.3
Gain/feed	0.75 ^{abc}	0.75 ^{bc}	0.73 ^{c}	0.75 ^{abc}	0.75 ^{abc}	0.78 ^{a}	0.76 ^{ab}	0.74 ^{bc}	0.74 ^{bc}	0.72 ^{c}	0.004
Kidney	9.1 ^{bc}	9.3 ^{bc}	9.5 ^{bc}	9.6 ^{ab}	8.6 ^{c}	9.4 ^{bc}	9.3 ^{bc}	10.4 ^{a}	9.0 ^{bc}	9.7 ^{ab}	0.11
Liver	27 ^{bc}	26 ^{bc}	28 ^{abc}	28 ^{abc}	25 ^{c}	29 ^{abc}	27 ^{bc}	29 ^{ab}	29 ^{ab}	30 ^{a}	0.40
Spleen	0.90	0.68	0.77	0.95	0.95	0.81	0.80	0.84	0.75	0.79	0.02
Bursa	2.50	2.09	2.16	2.07	2.35	2.23	2.12	2.55	2.34	2.36	0.07
Heart	5.54	5.84	5.90	5.64	5.55	5.62	5.35	6.17	5.52	5.86	0.11
Pancreas	3.78	3.72	4.04	3.65	3.99	4.00	3.49	3.83	4.01	3.94	0.08
Proventriculus	6.42	6.10	6.35	5.98	6.51	6.45	5.77	6.09	6.26	6.28	0.14
Gizzard F [1]	45	41	43	42	40	37	43	41	43	44	0.75
Gizzars E [1]	25	23	23	22	22	21	24	23	23	24	0.33

[a, b, c] Means within a row with different superscripts are significantly different ($P<0.05$).

[1] F – full; E – empty.

Table 4.4 One-way analysis of variance (ANOVA) using general linear model (GLM) with a priori contrast (P values).

ANOVA	Gain	Feed	Gain/ Feed	Kidney	Liver	Spleen	T3	T4	AME	Protein digesti.	SNP digestibility Ileal	SNP digestibility Fecal
GLM	ns [1]	ns	0.05* [2]	0.04*	0.09	ns	0.004** [3]	0.04*	0.0001**	0.01**	0.04*	0.001**
Plan. Contrast												
1 vs 2,3,4	ns	ns	ns	ns	ns	ns	ns	ns	0.003**	0.17	-	-
1 vs 5,6,7	ns	ns	ns	ns	ns	ns	ns	0.05*	0.002**	0.02*	-	-
1vs 8,9,10	0.12	0.18	ns	0.09	0.04*	ns	0.08	0.003*	ns	ns	-	-

ANOVA	Intestinal length				Intestinal full weight				Intestinal empty weight			
	Duode.	Jejunum	Ileum	Ceca	Duode.	Jejunum	Ileum	Ceca	Duode.	Jejunum	Ileum	Ceca
GLM	0.16	ns	ns	ns	0.09	0.19	0.002**	ns	ns	ns	ns	ns
Plan. Contrast												
1 vs 2,3,4	ns	ns	ns	ns	ns	ns	0.08	ns	ns	ns	ns	0.04*
1 vs 5,6,7	ns	ns	ns	ns	0.11	ns	0.18	ns	ns	ns	ns	0.05*
1vs 8,9,10	ns	0.13	ns	0.18	0.11	0.18	ns	ns	ns	ns	ns	0.004**

1 – control; 2, 3, 4 – treatments with three levels of SNP bisulfate; 5, 6, 7 – with SNP ethanol extract; 8, 9, 10 – with rapeseed meal.
[1] ns – not significant.
[2] * – significant, P<0.05.
[3] ** – very significant, P<0.01.
Duode. – duodenum; digesti. – digestibility.

Table 4.5 Regression analysis for control with SNP bisulfate, SNP ethanol extract, or with rapeseed meal treatments (P values).

Regression	Gain	Feed	Gain/ Feed	Kidney	Liver	Spleen	Gizzard full	Gizzard empty	T3	T4	AME	Protein digesti.
1, 2, 3, 4	ns [1]	ns	ns	ns	ns	ns	ns	0.09	ns	ns	ns	ns
Linear	ns	ns	ns	ns	ns	ns	ns	0.10	ns	ns	0.11	ns
Quadratic	ns	ns	ns	ns	ns	0.01**[3]	ns	ns	ns	ns	0.004**	ns
1, 5, 6, 7	ns	ns	0.18	ns	ns	ns	ns	0.17	ns	0.09	0.08	0.10
Linear	ns	ns	0.19	ns	ns	ns	ns	0.12	ns	0.11	0.01**	0.06
Quadratic	ns	ns	ns	0.11	ns	ns	0.15	0.05*	ns	ns	0.001**	0.03*
1, 8, 9, 10	0.09	0.19	0.14	ns	0.05* [2]	0.20	ns	ns	0.12	0.006**	ns	ns
Linear	0.10	0.20	0.14	ns	0.06	ns	ns	ns	0.13	0.006**	ns	ns
Quadratic	ns	ns	ns	0.20	ns	ns	ns	ns	ns	ns	0.01**	0.003**

Regression	Intestinal length				Intestinal full weight				Intestinal empty weight			
	Duode.	Jejunum	Ileum	Ceca	Duode.	Jejunum	Ileum	Ceca	Duode.	Jejunum	Ileum	Ceca
1, 2, 3, 4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Linear	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Quadratic	ns	ns	ns	ns	ns	0.06	0.11	ns	ns	ns	ns	0.16
1, 5, 6, 7	ns	ns	ns	ns	0.15	ns	0.14	ns	0.16	ns	ns	ns
Linear	ns	ns	ns	ns	0.17	ns	0.14	ns	0.17	ns	ns	ns
Quadratic	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1, 8, 9, 10	ns	0.13	ns	ns	ns	0.09	ns	ns	ns	ns	ns	0.008**
Linear	ns	0.14	ns	ns	ns	0.09	ns	ns	ns	ns	ns	0.01**
Quadratic	ns	ns	ns	ns	0.18	ns	ns	0.19	ns	ns	0.11	ns

1 – control; 2, 3, 4 – treatments with three levels of SNP bisulfate; 5, 6, 7 – with SNP ethanol extract; 8, 9, 10 – with rapeseed meal.
[1] ns – not significant.
[2] * – significant, P<0.05. [3] ** – very significant, P<0.01.
Duode. – duodenum; digesti. – digestibility.

The effects of dietary treatment on proportional tissue measurements are shown in Tables 4.3 and 4.6 with statistical interpretation in Tables 4.4 and 4.5. Chick kidney weight was affected by treatment as indicated by GLM analysis while a priori contrast demonstrated a numeric increase (P = 0.09) for diets containing RSM in comparison to the control. A quadratic relationship between control and level of SNP ethanol extract inclusion in kidney weight also approached significance (P = 0.11). Proportional liver weight was increased in diets only containing RSM but was unaffected by other dietary treatments in comparison to the control (a priori contrast, P=0.05). Spleen weight responded in a quadratic manner to the dietary inclusion of SNP bisulfate trihydrate with lower levels of addition resulting in reduced spleen weight and the highest level mean being similar to the control. Treatment did not affect bursa of Fabricius, heart and pancreas weights.

The empty gizzard weight showed a quadratic response to increased dietary inclusion of SNP ethanol extract with lower weights for lower levels (Table 4.3). Among other estimates of digestive tract size (length, full and empty weight), only the treatment effects on full ileum and empty ceca weights were significant (Table 4.6). Full ileum weight decreased in a quadratic manner in response to SNP bisulfate trihydrate. Empty cecal weight was smaller for all treatments in comparison to the control. In the case of RSM treatments, the empty ceca weight decreased in a linear fashion with inclusion of RSM (Table 4.5).

In comparison with the control, SNP bisulfate trihydrate increased diet AME (DM basis) in a quadratic fashion (Tables 4.5 and 4.7). A similar increase for AME and fecal protein digestibility was observed for the addition of SNP in the form of the ethanol

Table 4.6 The impact of SNP source and level on the relative length (cm/kg), and full and empty weights (g/kg) of the digestive tract (duodenum, jejunum, ileum, ceca) of broiler chickens.

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Canola meal			SEM
SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	
Duodenum L. [1]	45	41	45	42	41	47	46	48	46	45	0.69
Jejunum L.	92	92	99	92	90	95	97	103	97	105	1.59
Ileum L.	89	88	85	85	86	89	91	99	91	97	1.40
Ceca L.	38	37	40	37	38	39	37	41	38	41	0.51
Duodenum F. [1]	15	15	15	15	15	17	16	16	17	15	0.23
Jejunum F.	30	28	29	32	31	29	33	32	32	35	0.56
Ileum F.	25 ^{abc}	21 ^{d}	23 ^{bcd}	23 ^{bcd}	26 ^{ab}	22 ^{cd}	22 ^{cd}	28 ^{a}	25 ^{abcd}	28 ^{a}	0.51
Ceca F.	8.4	8.2	7.2	8.2	7.9	7.8	7.4	6.9	8.1	8.4	0.27
Duodenum E. [1]	13	12	13	13	13	15	13	13	15	13	0.27
Jejunum E.	20	18	18	19	20	20	20	20	19	19	0.32
Ileum E.	14	13	14	14	15	14	14	15	14	14	0.21
Ceca E.	5.8 ^{a}	4.4 ^{b}	4.7 ^{ab}	4.9 ^{ab}	4.6 ^{ab}	4.6 ^{ab}	5.1 ^{ab}	4.3 ^{b}	4.2 ^{b}	4.0 ^{b}	0.15

[a-d] Means within a row with different superscript are significantly different ($P<0.05$).

[1] L – length; F – full; E – empty.

Table 4.7 The effect of SNP source and level on the nutrient digestibility (AME (DM basis), apparent fecal protein digestibility), plasma triiodothyronine (T3) and thyroxine (T4) level, and apparent ileal and fecal digestibility of SNP in broiler chickens.

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Canola meal			SEM
SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	
AME (kcal/kg)	3279 ^{d}	3362 ^{bc}	3435 ^{a}	3325 ^{cd}	3428 ^{ab}	3377 ^{abc}	3330 ^{cd}	3396 ^{abc}	3276 ^{d}	3281 ^{d}	11.5
Fecal protein %	66.0 ^{bc}	67.0 ^{ab}	66.5 ^{abc}	66.8 ^{ab}	68.5 ^{a}	68.3 ^{a}	66.8 ^{ab}	68.3 ^{a}	65.3 ^{bc}	64.3 ^{c}	0.3
T3 (ng/dL)	174 ^{cd}	167 ^{cd}	159 ^{d}	176 ^{bcd}	149 ^{d}	219 ^{ab}	187 ^{bcd}	210 ^{abc}	168 ^{cd}	237 ^{a}	6.01
T4 (μg/dL)	0.48 ^{d}	0.56 ^{cd}	0.63 ^{bcd}	0.59 ^{cd}	0.72 ^{abcd}	0.57 ^{cd}	0.76 ^{abc}	0.67 ^{abcd}	0.86 ^{ab}	0.88 ^{a}	0.03
Ileal SNP %	-	0.396 ^{abc}	0.346 ^{bcd}	0.417 ^{ab}	0.379 ^{abc}	0.342 ^{bcd}	0.270 ^{d}	0.400 ^{abc}	0.294 ^{cd}	0.462 ^{a}	0.015
Fecal SNP %	-	0.716 ^{ab}	0.675 ^{bc}	0.689 ^{abc}	0.751 ^{a}	0.664 ^{bc}	0.654 ^{bc}	0.630 ^{c}	0.557 ^{d}	0.536 ^{d}	0.013
Ileal SA cont. %[1]	0	0.047	0.064	0.085	0.057	0.068	0.088	0.049	0.062	0.068	--
Fecal SA cont.%[1]	0	0.050	0.051	0.059	0.064	0.073	0.081	0.056	0.071	0.080	--

[a-d] Means within a row with a different superscript are significantly different ($P<0.05$).

[1] SA cont. % -- SA content was analyzed as % of dry ileal or fecal sample.

extract. Thus, both forms of SNP had a similar positive effect on the nutrient utilisation, as shown in Figure 4.1. Fecal digestibility of protein followed a quadratic pattern for RSM treatments with the lowest level of inclusion having improved digestibility but the higher levels having lower values than the control.

For plasma triiodothyronine (T3) and thyroxine (T4) measurement, treatments containing RSM tended to increase T3 levels in comparison to other diets (Tables 4.4 and 4.7). The T3 value for the RSM diets was numerically higher than the control but statistically not significant (P=0.08). For T4, both SNP ethanol extract and RSM treatments resulted in increased levels as compared to the control. The response of plasma T4 to RSM inclusion was linear (Table 4.7 and 4.5).

The ileal digestibility of SNP ranged from 35-42, 27-40, and 30-46 %, for SNP bisulfate trihydrate, SNP ethanol extract, and RSM treatments, respectively (Table 4.7). The fecal digestibility of SNP was 68-72, 65-75, and 54-63 %, for the same order of SNP source. The fecal SNP digestibility in RSM treatments was 10-15 % lower in comparison to the other treatments. In addition, treatments containing low levels of SNP had numerically higher fecal SNP digestibility than these with moderate or high levels of SNP.

SA was found in the ileum digesta and feces from birds in all levels of SNP bisulfate (Table 4.7) as well birds fed diets containing SNP and SA (ethanol extract and RSM treatments). SA was not found in the cecal digesta of any treatments.

4.6 Discussion

The analyzed protein, SNP and SA content for each treatment are shown in Table

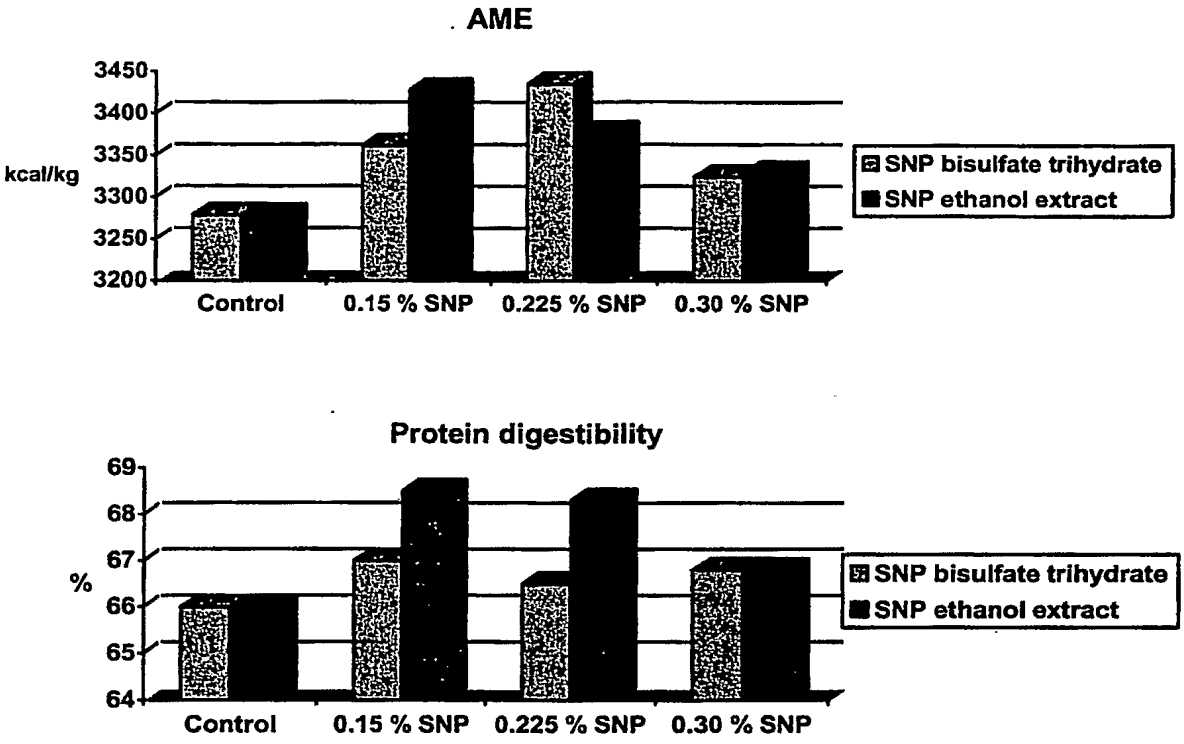


Figure 4.1 Effects of dietary SNP bisulfate trihydrate and SNP ethanol extract at three inclusion levels (as SNP %) on the diet AME and fecal protein digestibility of broiler chickens (Note: the analyzed values for SNP content (%) in practical diets are 0.124, 0.177, 0.249, and 0.094, 0.129, 0.158 for three levels of SNP bisulfate trihydrate and SNP ethanol extract treatments, respectively).

4.2. No major differences were observed in protein content among treatments indicating that diets were formulated and manufactured accurately. The analyzed dietary levels of SNP were lower than expected based on the experimental design. The actual values added should be accurate as all SNP sources were analyzed prior to diet preparation. It is therefore possible that the diet values reflected limitations of analysis technique, sampling error or the mixing capability of the feed mixer. The analyzed values were 75-80 % of added values for SNP bisulfate or RSM treatments, while the determined SNP content in ethanol extract was relatively lower. It is possible that the ethanol extracted phenolics bound or conjugated with other feed components thus interfering with the analysis. As expected both the ethanol extract and RSM diets contained SA. Thus, interpretation of the effects of these treatment groups must consider the presence of SA as well as other factors present in these impure sources of SNP. In the ethanol extract, minor biologically active components may exist such as flavanoids or trace amounts of tannins. In RSM, there are other anti-nutritional factors such as glucosinolates, phytic acid, tannins, and dietary fiber which may interfere interpretation of data based on SNP content.

Data reported in this research suggest that SNP included at levels up to approximately equivalent to that found in diets containing 30 % RSM do not cause toxicity or an anti-nutritional effect in broiler chickens. This statement is supported from the lack of a negative effect of dietary SNP bisulfate trihydrate on chick weight gain, feed consumption and the efficiency of feed utilization. Similarly, dietary SNP bisulfate trihydrate resulted in organ and digestive tract sizes equivalent to that of birds fed the corn-soybean meal based control diet. SNP in the semi-purified form of the concentrated

ethanol extract also did not affect chick growth related traits or for the most part organ and digestive tract size. Feeding RSM resulted in a numeric decrease in weight gain, but based on the results with SNP bisulfate trihydrate this is unrelated to diet SNP content. It is more likely that an adverse effect on bird performance could be attributed to the presence of anti-nutritional factors or the low digestibility of nutrients (e.g. amino acids) in RSM.

Purified or semi-purified SNP has rarely been used to study its effect in animal feeding and even in these trials, the data is less than conclusive. Austin et al. (1968) fed five *Sprague Dawley* rats diets containing 0.6 % SNP bisulfate for a period of seven days. They also found no effect on weight gain, feed consumption or feed efficiency, however the small number of animals used and the short duration of the study reduces the credibility of the results. In other research, Josefsson et al. (1976) carried out a study on eight 19-day-old specific pathogen-free NMRI male mice for eleven days based on a casein diet. They found that, SNP bisulfate added at the level higher than the SNP content in white mustard meal (2 %) resulted in a slower weight gain and a lower feed intake than the control group, but only the decrease in feed intake was significant. Feeding sodium bisulfate did not cause an effect thereby implicating SNP for the feed intake effect. However, the exact amount of "high level" of SNP bisulfate added in casein diet or the equivalent inclusion level of mustard meal in the ration was not described in this research and a previous (Josefsson et al., 1973) study. Thus, their results are confusing and questionable.

SNP is often suggested to be an important component of the reported low palatability of RSM (Josefsson et al., 1976; Sosulski, 1979; Ismail et al., 1981; Shahidi et

al., 1992). By tasting, the author confirmed the very bitter and sour flavor of SNP bisulfate trihydrate. The ethanol extract concentrate was bitter and sour, and also had a hot alcohol taste that may have been a reflection of residual ethanol. The author's observations indicate that these compounds could affect the flavor of RSM. Since feed consumption was not affected by SNP content in the current research, it would not appear to play an important role in determining the palatability of RSM for broiler chickens. It is also safe to assume that the exact effect of SNP on palatability of RS products is related to the diet inclusion level and the sensitivity of the targeted animal species.

Among control and SNP bisulfate treatments, there is a quadratic response in both the spleen and empty gizzard weights but the reason remains unknown. The demonstration of reduced empty cecal weight in all SNP treatments suggests that the hind gut might be one major metabolic site for these phenolic compounds in broiler chickens.

The present study provides evidence that dietary SNP bisulfate trihydrate, particularly at low levels, may provide a beneficial effect to broiler chickens. Inclusion of SNP bisulfate trihydrate increased diet AME by up to 4.8 %, an effect that closely resembled the response to feeding SNP in the ethanol extract concentrate. The latter treatment also increased fecal protein digestibility. It was noteworthy that inclusion of RSM at the lowest level of 15 % improved both diet AME and fecal protein digestibility in comparison to control, suggesting a similar beneficial effect possibly related to simple phenolics. Although these improvements did not result in improved performance, the magnitude of the response suggests this finding requires further exploration. Potential reasons for an improvement in nutrient utilization are not obvious. An inherent beneficial effect of SNP is possible but the potential for benefits as a result of hydrolysis to SA and

choline also exists. Low levels of SA may improve fecal protein digestibility (Chapter 5) and the release of SA in the small intestine is indicated by the fact that SNP digestibility by the end of the ileum ranges from 35 to 42 % for SNP bisulfate trihydrate treatments. Choline released in the small intestine is likely available for absorption but it is unlikely to benefit AME or protein digestibility as choline chloride was added to the diet in amounts that would readily meet the bird's requirements.

The disappearance of SNP in the avian digestive tract confirms previous research designed to investigate the effect of feeding RSM on egg flavor in laying hens (Fenwick et al., 1984). Although absorption of SNP can not be ruled out by this study, the fact that apparent digestibility is higher in feces than ileal contents, and the presence of SA in ileal and fecal samples from the SNP bisulfate trihydrate treatments (no SA in the diet) support the concept that the ester bond of SNP is hydrolyzed by gut micro-organisms to form SA and choline. It would appear that approximately half of the total SNP hydrolysis occurs before the end of the ileum while the cecal and colon environments are responsible for the remainder. The finding that SNP treatments uniformly decreased empty cecal weight also adds support for SNP or its metabolites having a presence in the avian hind gut.

Although SA was not found in the cecal digesta, it can be speculated that SA derived from SNP hydrolysis is either rapidly absorbed or transformed to other phenol metabolites which were not detected. This is supported from other research in this thesis (Chapter 5) demonstrating SA absorption in the small intestine. The latter study also failed to show SA in cecal contents but did show considerable SA in excreta suggesting absorption and then excretion in urine. The fact that TMA is produced from choline

derived from SNP in both the small intestine and ceca is also indirect evidence of the potential of bacteria to modify SA to a different form (Pearson et al., 1983; Fenwick et al., 1984). The only study conducted on the metabolism of SA in rats (Griffith, 1969) demonstrated that several metabolites derived from SA were found in the urine. Direct studies on other simple phenolic acids (caffeic acid, coumaric acid) in rats disclosed substantial modification of phenolic acid to other phenolic metabolites by gut bacteria (Scheline, 1968; Jung et al., 1983b).

It is of interest that the fecal SNP digestibility in RSM treatments was 10 to 15% lower than other treatments. This implies that components of RSM interfere with hydrolysis of SNP.

The majority of choline found in RSM exists in SNP. Therefore, its availability for absorption is likely determined by the degree of SNP hydrolysis and where the hydrolysis occurs. For the animal to derive benefit from RSM choline, it would appear that hydrolysis should occur in the small intestine and even there it is possible for it to be converted to TMA prior to absorption (Fenwick et al., 1984). Choline availability from RSM in avian species is reported to be half of that found in soybean meal (March et al., 1980). This may be explained by the low ileal SNP digestibility of RSM found in this research.

Feeding RSM in this study demonstrated a nearly significant reduction in growth rate (planned contrast, $P=0.12$), an increase in liver weight and increases in plasma T3 (GLM, $P<0.01$; planned contrast, $P=0.08$) and T4. These effects were not found when purified SNP bisulfate trihydrate was fed and therefore these effects must be attributed to other factors in the meal. In the case of liver weight, other studies have suggested

hydrolysis products of glucosinolates, especially nitriles, cause liver enlargement and lesions (Bille et al., 1983; Bjergegaard et al., 1994; Thomke et al., 1998). The current study indicates that residual glucosinolates in RSM might still have a negative impact on liver function. The impact of glucosinolate hydrolysis products (especially oxazolidinethione) on thyroid metabolism has long been known (Christison and Laarveld, 1980; Liu et al., 1994; Thomke et al., 1998) but the increase in plasma hormones might be considered unexpected since the above anti-nutrients are known to cause hypothyroidism. However our finding agrees with Schone et al. (1993). They suggested that a higher serum T3 concentration could occur in strongly hypothyroid chicks. Anti-thyroid compounds could destroy cellular T3 receptors and the impaired T3 transfer might cause an increase in blood hormone concentration. Other recent work in our lab also found elevated serum T3 levels in broilers fed graded dietary levels of canola meal or white-flake, as compared to a corn-soybean control diet (Newkirk and Classen, 2001). The fact that SNP ethanol extract also increased plasma T4 is of interest and may offer an opportunity to purify the agent responsible or partially responsible for the effect.

SA was found in both the ethanol extract and RSM diet. This is expected based on previous research showing the ethanol solubility of SA and free SA in RSM. Establishing the apparent digestibility of SA is difficult because one must consider not only the amount in the original diet but also the SA released from SNP hydrolysis. For more detailed research on the digestibility of SA, see Chapter 5.

4.7 Conclusion

This is the first systematic study to clarify the effects of sinapine in broiler chickens using purified SNP derived from RSM. Dietary sinapine contributes to the bitter and sour tastes of RSM, however, at the levels fed in this research SNP (0.15 to 0.30 %) did not demonstrate toxic or anti-nutritional effects in broiler chickens. Therefore, even at the levels of SNP that approximate levels found in diets containing 30 % RSM, diet palatability for broiler chickens was not affected. In contradiction to the commonly held belief that SNP is an anti-nutritional factor in RSM, it was found the SNP in purified or semi-purified form improved nutrient utilization at low levels of inclusion. Relatively minor effects of SNP on organ weights also support the idea that SNP is not toxic at these dietary levels. The reduced empty weight of ceca in all SNP treatments suggests that the hind gut might be a major metabolic site for SNP.

SNP digestibility was found to range from 27-46 % in the ileum and 54-75 % in excreta. One major metabolic pathway of SNP is via hydrolysis into sinapic acid and choline, probably due to gut bacteria. Purified SNP caused effects which were similar to that of its semi-purified form, from a nutritional, physiological and metabolic perspective.

Feeding RSM was found to increase relative liver weight and plasma T4 and T3 independent of an effect of SNP.

5. NUTRITIONAL, PHYSIOLOGICAL AND METABOLIC EFFECTS OF DIETARY SINAPIC ACID IN BROILER CHICKENS

5.1 Abstract

Sinapic acid (SA) is a major free phenolic acid in rapeseed meal with the majority found in the esterified form of sinapine. Two experiments were conducted to delineate the effect of dietary SA on broiler chickens, in terms of performance, nutrient digestibility and toxicity, and also to examine the metabolism of SA in the digestive tract. In the first experiment, 80 male broiler chicks (*Peterson* X *Hubbard*) were randomly assigned to five treatments containing 0, 0.05, 0.10, 0.15 or 0.20 % SA. Performance from 0 to 18 d of age, and the relative size of the bursa of Fabricius, heart, kidney, liver, spleen, pancreas, proventriculus, gizzard, duodenum, jejunum, ileum and ceca were not affected by dietary treatment (all $P>0.05$). In addition, dietary SA had no effect on the activity of creatine kinase and lactate dehydrogenase in serum ($P>0.05$).

The objectives of the second experiment were to confirm the results of the first trial, and to investigate the impact of SA on nutrient digestibility, and to study the metabolism of SA in the digestive tract. Male broiler chicks (96) were randomly assigned into four treatments containing 0, 0.025, 0.05, and 0.10 % SA. Dietary SA at the 0.025 % level increased feed intake in comparison to control ($P<0.05$). Regression analysis indicated a quadratic response for both weight gain and feed intake ($P<0.03$ and 0.01), as SA increased in the diet with the highest values associated with the lowest level of SA. Treatment did not affect feed efficiency. Again dietary SA did not affect bursa of

Fabricius, liver, kidney or digestive tract size. AME and protein digestibility were not affected by SA level. However, regression analysis indicated a nearly significant linear increase in AME (P=0.08) and decrease in ileal protein digestibility (P=0.07) with increasing SA level.

Apparent ileal SA digestibility was above 97 % for all levels of inclusion, whereas the fecal SA digestibility ranged from 64 to 79 %. This suggests that a high proportion of dietary SA is absorbed prior to the ileum, and 20-35 % of the SA is excreted in its original form into the urine. In summary, SA at dietary levels used in this study does not have toxicity or anti-nutritional effects in broiler chickens. Dietary SA level at 0.025 % increased broiler feed intake and growth performance.

(*Key words*: sinapic acid, rapeseed meal, toxicity, digestibility, metabolism)

5.2 Introduction

Sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid) is the predominant free phenolic acid found in rapeseed. Sinapic acid (SA) constitutes up to 15 % of total phenolics in rapeseed meal (RSM) and up to 99 % of phenolic acids released from esters and glucosides in rapeseed flours (Krygier et al., 1982). It can be estimated that the free SA content in RSM may range from 0.05 to 0.4 % or higher depending on the variety, and growing location and conditions (Krygier et al., 1982; Shahidi et al., 1992). A large proportion of SA is found as a portion of the compound, sinapine. Sinapine (SNP) is the major phenolic compound in RSM, and is comprised of SA esterified to choline. As with SNP, little research has been done on SA in RSM from either a chemical or biological standpoint.

It has long been recognized that compounds in the 4-hydroxy-3,5-dimethoxy-phenyl group occur widely in dietary material of plant origin. Dietary constituents of this type may give rise to metabolites possessing undesirable psychopharmacological properties, especially in schizophrenic individuals where abnormalities of methylation may exist (Kety, 1965). In animal nutrition, it has been reported that phenolic compounds in RSM, mainly SNP and SA, may contribute to the dark color, bitter taste and astringency of the meal (Sosulski et al., 1977, 1979; Shahidi et al., 1995; Naczk, et al., 1998). Therefore, they are thought to be responsible for palatability problems associated with feeding RSM to pigs and rats (Josefsson et al., 1976; Lee et al., 1984). They are also frequently reported to be responsible for the deleterious properties of protein products derived from rapeseed (Rutkowski, 1979). During protein isolation

under alkaline conditions, it is possible that SNP could be hydrolyzed into SA and choline, which both have bitter tastes.

Phenolic compounds and/or their derived products have the potential to form complexes with essential amino acids and digestive enzymes, thus lowering the nutritional value of rapeseed products. Some *in vitro* studies have already demonstrated the evidence (Wada et al., 1969; Rutkowski et al., 1977; Ismail, 1981; Lee et al., 1984; Naczk et al., 1998). Recently, SA from RSM was demonstrated to bind with bovine serum albumin *in vitro*, and therefore has the potential to decrease the nutritional value of RSM as a protein source (Shahidi et al., 1995). It is not known if SA can bind with nutrients other than protein. SA has also been shown to have strong antioxidative (Nowak et al., 1992; Wanasundara et al., 1996), and antibacterial activity *in vitro* (Nowak et al., 1992; Tesaki et al., 1998; Hua et al., 1999).

Although the concentration of SA in free form in RSM is relatively small, the potential for larger amounts of SA to be released from SNP suggests that specific knowledge on the nutritional and toxicological role of SA in animal feeding is required. Research on the impact of dietary SA on non-ruminant (Griffiths, 1969) or ruminant animals is very scarce. Two experiments were conducted to study the impact of dietary SA on broiler chickens. The objectives of the first experiment were to investigate nutritional and toxicological effects of SA. The second experiment was conducted to confirm the results of the first trial, to investigate the impact of SA on nutrient digestibility, and to study the metabolism of SA in the digestive tract of broilers.

5.3 Materials and Methods

5.3.1 Experimental design

Experiment 1. Five treatments consisted of corn soybean meal diets supplemented with graded levels of dietary SA (0, 0.05, 0.10, 0.15 and 0.20 %). SA addition was equivalent to the SA profiles of sinapine when RSM is added at 7.5, 15.0, 22.5 and 30.0 % in the formula diet, if the actual SNP content in RSM was estimated at 1.0 %. It can be speculated that the dietary SA level at 0.05 % is equivalent or higher than the free SA content of a diet containing 30 % RSM. Each diet was replicated four times with four birds per replication. A total of 80 one-day-old male broiler chicks (*Peterson* X *Hubbard*) were randomly assigned to experimental units.

Experiment 2. Four treatments were based on a corn-soybean meal diet with graded levels of dietary SA (0, 0.025, 0.05, and 0.10 %). Male broiler chicks (*Peterson* X *Hubbard*) were randomly assigned into replication groups containing six birds each, and four replications were used for each treatment.

5.3.2 Bird management

Broilers were housed in battery brooders. Temperature was maintained in accordance with standard brooding management and light was provided for 23 h and 16 h from 0 to 5 and 5 to 18 d of age, respectively. Feed, in mash form, and water were provided *ad libitum.* SA was purchased from Sigma® Chemical Co. (P.O. Box 14508 St. Louis, MO 63178 USA) with a purity of 98 % (GC grade, Lot 128H3485, light yellow or milky color, dry powder) and was diluted with corn prior to feed manufacture. Diets

were formulated to be isoenergetic and isonitrogenous, and either meet or exceed the nutrient requirements of broiler chicks as recommended by the National Research Council (1994). Diet composition and nutrient levels are shown in Table 5.1.

5.3.3 Data Collection

Experiment 1. Weight gain and feed consumption for each pen were measured at 7, 14 and 18 d of age while mortality was recorded and weighed daily. At the end of 18 d, the birds were individually weighed, blood sampled to provide the serum for analysis of creatine kinase (CK) and lactate dehydrogenase (LD), killed by lethal injection with T-61®(Euthanasia solution), and internal organs (bursa of Fabricius, heart, kidney, liver, spleen, pancreas, proventriculus, gizzard) and intestines (duodenum, jejunum, ileum and ceca) were removed and measured. For each section of the digestive tract, both full and empty weights were measured. Since CK is primarily located in skeletal muscle, brain tissue and heart muscle, whereas heart, kidney, liver, brain, erythrocytes and skeletal muscle have high percentages of LD, damage to these tissues results in the release of increased levels of CK and LD into blood.

Experiment 2. Production parameters were monitored from 0 to 18 d of age. Excreta were collected for each pen from 16 to 18 d of age. At the end of 18 d, the birds were individually weighed and killed by chemical agent (T-61®). Organs and digestive tracts were removed, and the bursa of Fabricius, liver, kidney, ileum and ceca were measured. Ileal and cecal digesta were collected from each bird and pooled together within pen, and immediately frozen at –20 °C until further analysis. All other aspects of the trial were as described for Experiment 1.

Table 5.1 Dietary composition[1] and calculated major nutrient level (Experiments 1 & 2).

Diet Composition	Exp. 1 %	Exp. 2 %	Nutrient Level	Exp. 1 %	Exp. 2
Corn	55.70	56.61	AME (kcal/g)	3.10	3.10
Soybean meal	34.86	34.62	Crude protein	21.00	21.00
Canola oil	3.59	2.82	Calcium	0.95	0.95
Dicalcium phosphate	1.76	1.56	Non-phytate P	0.45	0.45
Limestone	1.20	1.60	Linoleic acid	1.87	1.77
Sodium chloride	0.46	0.46	Arginine	1.38	1.38
Choline chloride	0.10	0.10	Lysine	1.20	1.20
Vit./Min. premix [2]	0.50	0.50	Methionine	0.56	0.56
DL-Methionine	0.24	0.24	Methionine + cystine	0.90	0.90
L-Lysine HCl	0.10	--	Threonine	0.80	0.81
Celite	1.50	1.50	Tryptophan	0.26	0.26

[1] Sinapic acid (98 %) was added at 0, 0.05, 0.10, 0.15 and 0.20 % in Experiment 1 and at 0, 0.025, 0.05, and 0.10 % in Experiment 2.

[2] Vitamin and mineral premix supplied per kilogram of diet: vitamin A (retinyl acatate + retinyl palmitate), 11000 IU; vitamin D_3, 2200 IU; vitamin E (dl-α-topheryl acetate), 300 IU; menadione, 2.0 mg; thiamine, 1.5 mg; riboflavin, 6.0 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10.0 mg; folic acid, 0.6 mg; and biotin, 0.15 mg; ethoxyquin, 0.625 mg; iron, 80 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; and selenium, 0.3 mg; calcium carbonate, 500 mg.

5.3.4 Sample analyses

Creatine kinase (CK) and lactate dehydrogenase (LD) activity were measured in serum at 340 nm by spectrophotometry according to the Sigma Diagnostics® Kits for CK and LD (Sigma Chemical Co., St. Louis, MO, USA). Sigma LD reagent measures the LD activity based on the oxidation of lactate to pyruvate. The assay for CK was based on Szasz's procedure which established optimal conditions for CK measurement.

Acid insoluble ash (AIA, Celite) was used as an indigestible marker for the determination of the apparent digestibility of metabolizable energy, protein (ileal and fecal) and SA. AIA was determined by using the procedure of Vogtmann et al. (1975). The gross energy was measured by a traditional bomb calorimeter (AOAC, 1990) and crude protein was analyzed by a Leco FP-528 protein analyzer (Model No. 601-500-100, Serial # 3211, LECO Corporation, 3000 Lakeview Avenue, St. Joseph MI 49085-2396 USA). Based on the assay of nutrient and AIA in feed, excreta, and ileal content, the nutrient digestibility was determined.

The analysis of SA in feed, excreta and digesta samples is described in Chapter 3.2. Sample preparation was the same as in SNP assay (Ch 3.2.2.2). The analysis was conducted by HPLC using a reversed-phase column under the fluorescence detection method. The mobile phase was isocratic, based on 6 % MeOH solution with 20 mmol K_2HPO_4 as basic buffer (Table 3.1). For feed and excreta samples, the analysis was conducted at pH 9.5, however, for ileal and cecal samples, the pH was changed to 9 to obtain better background separation and chromatograms.

5.4 Statistical analyses

Data for tissue weights and/or lengths were based on bird weight value/body weight. All data were subjected to one-way Analysis of Variance (ANOVA) according to the General Linear Model (GLM) procedure and a priori contrast, as well regression analysis by the SAS program (SAS® Institute, 1999). Differences were considered significant when $P<0.05$, unless otherwise stated.

5.5 Results

5.5.1 Experiment 1

There were no differences among dietary SA treatments with respect to the body weight gain, feed consumption, and feed efficiency (gain to feed ratio) (all $P>0.05$, see Table 5.2).

No differences were observed among the five treatments in the relative weight of the bursa of Fabricius, heart, liver, kidney, spleen or pancreas (Table 5.2). Similarly, treatment did not affect relative weight of the proventriculus, and gizzard, and the length, full, and empty weights of duodenum, jejunum, or ceca. Dietary treatment had minor effects on empty ileum weight.

The effect of dietary SA on broiler serum creatine kinase (CK) and lactate dehydrogenase (LD) activities are shown in Table 5.2. There were no treatment effects. There was a trend for the activity of CK to decrease as SA level increased, but this observation is statistically not significant.

Table 5.2 Effects of dietary SA on the performance, relative internal organ weight and intestinal measurement, and toxicity of broiler chickens (Experiment 1).

Treatment	Sinapic acid (%)					SEM	GLM	Contrast	Regression	
	0	0.05	0.10	0.15	0.20			1 vs 2-5	Linear	Quadratic
Gain (g)	481	500	481	511	499	8.9	ns	ns	ns	ns
Feed intake (g)	694	706	689	721	703	9.1	ns	ns	ns	ns
Gain/feed	0.70	0.71	0.70	0.71	0.71	0.005	ns	ns	ns	ns
Bursa (g/kg)	2.5	2.4	2.7	2.3	2.5	0.059	ns	ns	ns	ns
Heart (g/kg)	6.2	6.1	6.4	6.5	6.3	0.060	ns	ns	ns	ns
Kidney (g/kg)	8.8	9.2	9.4	9.3	9.1	0.11	ns	ns	ns	ns
Liver (g/kg)	32	33	33	32	31	0.29	ns	ns	ns	ns
Spleen (g/kg)	0.98	0.87	0.90	1.06	1.03	0.023	ns	ns	ns	ns
Pancreas (g/kg)	4.2	3.7	3.9	4.3	3.6	0.073	ns	ns	ns	ns
Proventri. (g/kg)	7.1	7.0	7.0	7.3	6.8	0.063	ns	ns	ns	ns
Gizzard F.(g/kg)	48	46	47	47	46	0.43	ns	ns	ns	ns
Gizzard E. (g/kg)	28.6	27.7	27.9	27.9	27.7	0.22	ns	ns	ns	ns
Duode. L.(cm/kg)	46	42	44	45	43	0.82	ns	ns	ns	ns
Jejun. L. (cm/kg)	107	105	103	106	101	1.62	ns	ns	ns	ns
Ileum L. (cm/kg)	96	91	100	99	96	1.66	ns	ns	ns	ns
Ceca L. (cm/kg)	41	39	41	40	41	0.66	ns	ns	ns	ns
Duode. F. (g/kg)	15	14	14	15	14	0.28	ns	ns	ns	ns
Jejun. F. (g/kg)	32	32	31	32	30	0.56	ns	ns	ns	ns
Ileum F. (g/kg)	25	26	25	29	24	0.58	ns	ns	ns	ns
Ceca F. (g/kg)	8.2	7.8	7.5	7.6	8.8	0.31	ns	ns	ns	ns
Duode. E. (g/kg)	13	12	12	12	12	0.30	ns	ns	ns	ns
Jejun. E. (g/kg)	21	18	19	21	19	0.57	ns	ns	ns	ns
Ileum E. (g/kg)	15	14	14	16	14	0.24	0.03*	ns	ns	ns
Ceca E. (g/kg)	5.1	5.0	5.0	4.8	5.3	0.13	ns	ns	ns	ns
CK[1] (RA)	0.082	0.075	0.081	0.064	0.060	0.008	ns	ns	ns	ns
LD[1] (RA)	0.055	0.057	0.060	0.049	0.062	0.005	ns	ns	ns	ns

L – length, F – full, E – empty, RA – relative activity unit. [1] CK – creatine kinase, LD – lactate dehydrogenase. * $P<0.05$.

5.5.2 Experiment 2

Body weight gain responded in a quadratic manner to increasing levels of dietary SA (GLM, P=0.12; quadratic regression, P=0.03) as did feed consumption (quadratic regression, P<0.01) (Table 5.3). Both weight gain and feed intake were highest at the lowest SA level of 0.025 % and declined to near control values for the highest level of SA inclusion at 0.10 %. Feed efficiency was not affected by treatment.

There were no differences among treatments in the relative weight of the bursa of Fabricicus, kidney and liver, and the relative weight and length of the full and empty ileum (Table 5.3). Cecal length and full weight were not affected by dietary SA but empty cecal weight decreased with increasing levels of SA (linear regression, P=0.04).

AME was not affected by dietary treatment, however, the AME at all SA levels were numerically higher than control and regression analysis indicated a linear increase with increasing SA level, which was close to significance (P=0.08) (Table 5.3). The effect of SA on ileal protein digestibility was also not significant by GLM analysis but regression indicated a nearly significant linear decrease with increasing SA level (P=0.07). Fecal protein digestibility followed the same pattern as AME with dietary SA treatments, however, the numerically highest fecal protein digestibility was found in treatment of the lowest SA level at 0.025 %.

A high proportion of SA disappeared prior to the ileum as shown by apparent ileal digestibility values between 97.0 and 97.8 % for the three SA diets (Table 5.3). Significant differences were observed for fecal SA digestibility among dietary SA treatments (P<0.001). The lowest level of SA at 0.025 % had the highest value for fecal digestibility. Values for all treatments ranged from 63.8 to 79.3 %. There was no SA

Table 5.3 Effects of dietary SA on the performance, relative internal organ weight and intestinal measurement, and apparent nutrient and SA digestibility in broiler chickens (Experiment 2).

Treatment	Sinapic acid (%)				SEM	GLM	Contrast	Regression	
	0	0.025	0.05	0.10			1 vs 2,3,4	Linear	Quadratic
Gain (g)	522	561	545	491	11.2	0.12	ns	ns	0.03 *
Feed intake /bird (g)	718 [b]	781 [a]	738 [ab]	689 [b]	11.8	0.02 * [1]	ns	0.13	0.01 ** [2]
Gain/feed	0.73	0.72	0.74	0.71	0.01	ns	ns	ns	ns
Bursa (g/kg)	2.32	2.29	2.46	2.29	0.09	ns	ns	ns	ns
Kidney (g/kg)	10.4	10.0	10.5	10.2	0.15	ns	ns	ns	ns
Liver (g/kg)	35.4	34.5	33.6	34.2	0.56	ns	ns	ns	ns
Ileum L. (cm/kg)	93.4	84.2	92.0	94.5	1.90	ns	ns	ns	ns
Ceca L. (cm/kg)	38.9	40.6	41.0	40.4	0.57	ns	ns	ns	ns
Ileum F. (g/kg)	25.7	24.6	27.0	24.5	0.65	ns	ns	ns	ns
Ceca F. (g/kg)	9.8	10.1	11.2	9.5	0.43	ns	ns	ns	ns
Ileum E. (g/kg)	14.5	14.1	14.7	14.4	0.18	ns	ns	ns	ns
Ceca E. (g/kg)	6.1	6.2	5.9	5.4	0.14	0.13	ns	0.04 *	ns
AME (kcal/kg)	3348	3412	3418	3442	17.4	ns (0.27)	0.07	0.08	ns
Ileal protein digest. %	0.825	0.815	0.788	0.800	0.006	ns (0.16)	0.09	0.07	ns
Fecal protein digest.%	0.648	0.685	0.663	0.665	0.007	ns (0.31)	ns (0.16)	ns	0.21
Ileal SA digest. %	-	0.970	0.970	0.978	0.002	0.14	-	-	-
Fecal SA digest. %	-	0.793 [a]	0.638 [c]	0.713 [b]	0.020	0.0001**	-	-	-

[a, b, c] Values with different letters of superscript in the same row are significantly different (P<0.05).

[1,2] *- P<0.05, ** - P<0.01.

SEM – standard error of means, L – length, F – full weight, E – empty weight, digest. – digestibility.

found in the cecal digesta.

5.6 Discussion

Dietary sinapic acid at levels that approximate that found when more than 30 % RSM is added in the diet did not cause obvious anti-nutritional effects on broiler chickens. In both experiments, dietary SA did not reduce growth rate, feed consumption or gain to feed ratio. In fact, in Experiment 2 the lowest level of SA increased both growth rate and feed intake. The latter effect appears to be in contrast to the frequently cited and obvious bitter taste and astringency of SA (Ismail et al., 1981; Haslam, 1988). However, the relatively low level of dietary inclusion may explain the lack of a negative palatability effect. The current study found that none of the internal organs or intestinal sections measured, including the liver and kidney which may participate in phenolic metabolism and excretion, were affected by dietary SA. Failure to demonstrate an increase in serum CK and LD activity adds further support for a lack of a negative effect of SA. These data suggest that dietary SA is not toxic in broiler chicks from nutritional and physiological standpoints.

The results in the current research are in agreement with limited research on feeding purified SA to rats. Griffiths (1969) administered SA orally to rats (200 mg per animal in the diet, body weight 250 g) in single doses in an admixture with a standard rat diet for five days and found no effect of treatment on growth parameters although SA and several phenolic metabolites were excreted in the urine. The relatively small number of animals used and short duration of the experiment reduces the reliability of these data.

A potential beneficial effect of dietary SA, as indicated by growth rate and feed intake, may be associated with the antioxidant activity of SA and related plant phenolics. For example, ferulic acid has been claimed to lessen the effects of chemo- and radiotherapy of carcinomas by increasing the natural immune defense (Graf, 1992). Other beneficial effects of ferulic acid include strong anti-inflammatory properties in a rat paw edema model, inhibition of chemically-induced carcinogenesis in rats and tumor growth in mouse skin (Graf, 1992). Although research has rarely been done using SA, the similar chemical structure of ferulic acid legitimizes a comparison. Ferulic acid is a precursor in SA synthesis, and both phenolic acids have a hydroxy group attached to the 4- position of the benzene ring, a methoxy group at the 5- position and a double bond between the same carbons of the side chain. It is of interest that SA has strong antioxidant activity *in vitro* (Nowak et al., 1992). Also similar to ferulic acid, is the more recent *in vitro* demonstration that SA has antibacterial activity (Tesaki et al., 1998). It is of interest that empty cecal weight was reduced in response to increasing dietary SA in one of the two experiments in the current research. This is similar to the response noted for SNP in Chapter 4.

SA was nearly absent from the terminal end of the ileum and as a consequence apparent digestibility values were very high (97.0 to 97.8 %). In contrast, apparent fecal digestibility values were much lower (63.8 to 79.3 %). These data suggest that SA is readily absorbed prior to the terminal ileum and that at least a portion of the SA was excreted intact via the kidney. The latter is in agreement with the only report on SA metabolism study in rats which demonstrated the excretion of a certain amount of intact form of SA in the urine (Griffiths, 1969). Up to now, no research has examined SA

absorption in the gut of animals but work with other phenolic acids (cinnamic, caffeic, and ferulic acid) has shown substantial absorption in the small intestine (Jung et al., 1983b; Woffram et al., 1995; Olthof et al., 2001). However, after absorption, the metabolic fate of these phenolic compounds, and their potential toxicological or biological effects on animals are not known. With respect to excretion, research has suggested that ingestion of plant phenolics results in a considerable increase in urinary excretion of phenolics and that most of the absorbed phenolics are metabolically transformed before being excreted (Martin, 1982; Jung et al., 1983b; Silanikove et al., 1989; Arin et al., 1992). In a study using rats, radiolabeled cinnamic acid was used as a model for monomeric cinnamic acid derivatives to study jejunal absorption mechanisms (Woffram et al., 1995). They demonstrated the involvement of a sodium-dependent, carrier-mediated transport process across the brush border membrane for cinnamic acid and structurally related substances such as ferulic acid. Other research has also indicated that intestinal microflora can modify phenolic compounds in the digestive tract (Jung et al., 1983a). It is probable that gut microorganisms have modified a portion of dietary SA in the current research. The impact of SA on empty cecal weight, and the failure to find SA in the ceca of birds fed either SA or SNP (see Chapter 4) support this conclusion. It is unknown whether dietary SA, to a lesser extent, could also be transformed to other metabolic forms or absorbed prior to access into the small intestine. A comprehensive study capable of monitoring gut and post absorption modification of SA, and identification of resulting compounds is required to fully understand *in vivo* SA metabolism and its impact on the host animal.

Cinnamic acid and its derivatives, including SA, are directly involved in the synthesis of polymeric plant phenols such as lignin and flavonoids (Jung et al., 1983a) and thus may contribute to the indigestible property of fibrous components of RSM. Since SA is capable of binding protein *in vitro* (Kozlowska et al., 1990), it was of interest to see if dietary SA affects protein digestion and absorption. Apparently contradictory evidence was found in this study. Apparent ileal protein digestibility decreased in a linear fashion while fecal protein digestibility was 6 % higher for the lowest level of dietary SA (0.025%) in comparison to control diet. The latter response corresponds well with increased growth rate (7.5 %) in this treatment and a numeric increase in AME (2 %). The linear decreased trend in ileal protein digestibility may be explained by the *in vitro* evidence of the binding property of SA. SA may bind with the diet proteins by decreasing protein digestibility. It is also possible that SA may bind with proteins in the host animal, such as digestive enzymes, which in turn interfere with protein digestion and absorption, especially at high dietary SA levels. The much higher SA digestibility found in the ileum than in excreta, suggests that dietary SA, after significant absorption in the small intestine, may also have the impact on the systematic circulation of animal host possibly by its antioxidant and antibacterial potential. The data shown in this study may be a reflection of compromise between the potential negative and positive effects of dietary SA in broiler chickens. The demonstrated quadratic effect of dietary SA in Experiment 2 indicates that there may be a fine line between the two sides of SA effect.

The increased growth rate and feed intake, and improved apparent nutrient digestibility at 0.025 % SA suggest that SA might be considered as a growth promotant in non-ruminant animal diets. This experiment was conducted in relatively clean battery

cages and excellent environmental conditions, therefore a growth response would not be expected. Under commercial extensive animal production environments, usually a more significant growth promotion effect is anticipated when using growth promotants. Therefore, the improved growth performance in the current research supports the idea that SA might be a candidate as a growth promotant or a natural alternative to antibiotics in non-ruminant animal production.

5.7 Conclusion

Dietary SA did not decrease weight gain, feed consumption, and gain to feed ratio when added at levels that approximate the total SA in diets containing 30 % RSM. Since SA did not affect feed intake in broiler chickens, it does not appear to be responsible for the palatability problems reported for RSM. On the contrary, a low level of dietary SA level at 0.025 % may stimulate feed intake and improve the performance of broiler chickens. Other indications that SA is not toxic or an anti-nutritional factor in broiler chickens is the lack of treatment effect on the relative weight and measurement of key internal and digestive organs.

Higher levels of dietary SA (0.05 and 0.10 %) may interfere with protein digestion and utilization in ileum. This characteristic did not affect AME. However, dietary SA level at 0.025 % showed improvement in fecal protein digestibility, which suggests the involvement of hind gut, possibly through the participation of hind gut bacteria.

SA digestibility was around 97 % for all levels in the ileum, whereas the fecal SA digestibility approximated 64-79 %. The disappearance of SA in the ileal digesta infers that it is mainly absorbed in the small intestines of broiler chickens, and that after systemic metabolism at least a portion (20-35 %) is excreted in its original form into urine. The potential conversion of SA into other metabolites by gut microbes or detoxification mechanisms of the bird can not be ruled out since metabolites were not measured in this research.

6. THE EFFECT OF RAPESEED MEAL SIMPLE PHENOLICS ON VOLATILE FATTY ACID PRODUCTION AND RELATED ANTIBACTERIAL ACTIVITY IN BROILER CHICKENS

6.1 Abstract

Sinapic acid (SA) is a major phenolic acid in rapeseed meal (RSM) where large amounts are found in the esterified form of sinapine (SNP). To delineate the antibacterial effect of RSM simple phenolics on broiler chickens three experiments were conducted to study their effects on volatile fatty acid (VFA) production and the microbial profile in the ileum and ceca. The first experiment was designed to study the effect of RSM sinapine in pure (SNP bisulfate), semi-purified (ethanol extract), or native form in RSM on VFA production in the ileum and ceca of broiler chickens. Male broilers (120, *Peterson X Hubbard*) were assigned to ten treatments. The net SNP content of three levels of SNP bisulfate or ethanol extract concentrate were equivalent to the SNP profile in diets with increasing levels of RSM at levels of 15.0, 22.5, and 30.0 % inclusion. The performance was monitored from 0 to 18 days after which the chicks were killed, and ileal and cecal digesta collected for VFA measurement. SNP bisulfate trihydrate increased the ileal acetic acid and total VFA levels with a quadratic response (all $P<0.05$), as well isovaleric and butyric acid levels in a positive linear fashion. Cecal VFA were not affected by any dietary SNP treatment ($P>0.05$).

Experiments 2 and 3 evaluated the effect of graded levels of SA on VFA levels in ileal and cecal contents. SA was fed at 0, 0.05, 0.10, 0.15 and 0.20 % and 0, 0.025, 0.05

and 0.10 % in experiments 2 and 3, respectively. In both SA trials VFA (especially acetic acid) levels increased in the ileum in a linear fashion ($P<0.05$) with SA levels, but decreased the acetic acid and total VFA levels (including acetic, propionic, butyric, isobutyric, valeric, and isovaleric acid) by 10-30 % in ceca of broiler chicks (both $P<0.01$). The level of total VFA in ileum was much lower than in ceca. Data suggest that SA might have a strong antibacterial activity *in vivo*. Further microbial analysis by percentage-guanine-plus cytosine disclosed that dietary SA may cause a shift in the populations of cecal bacteria without changing the total number of cecal bacteria.

(*Key words*: sinapic acid, volatile fatty acid, rapeseed/canola, percentage of guanine plus cytosine, broiler chicks)

6.2 Introduction

Sinapic acid (4-hydroxy-3, 5-dimethoxy-cinnamic acid) is a predominant phenolic acid found in rapeseed. Sinapic acid (SA) constitutes up to 15 % of total phenolics in rapseseed meal (RSM) and up to 99 % of phenolic acids released from esters and glucosides in the canola flours (Krygier et al., 1982). Free SA content in RSM is estimated to range from 0.05 to 0.40 %. Large amounts of SA are found as a portion of the choline ester, sinapine (SNP). SNP is the major phenolic compound in RSM. Both SNP and SA are simple phenolics in RSM in comparison to polyphenol tannins.

SNP and SA have been considered anti-nutritional factors because they are believed to contribute to the deleterious organoleptic properties of RSM, and may affect protein digestibility *in vitro* (Josefsson et al., 1976; Rutkowski et al., 1977; Sosulski et al., 1979; Ismail, 1981; Lee et al., 1984; Shahidi et al., 1995; Naczk et al., 1998). Microbial hydrolysis of SNP in the gastrointestinal tract of chickens has also been shown to release choline which in turn is converted to trimethylamine (TMA) which is absorbed. In some breeds of chickens, a lack of TMA oxidase activity results in absorbed TMA being deposited in eggs rather than being excreted in urine as TMA oxide. The presence of TMA results in a fishy taint in eggs. However, research in this thesis (Chapters 4 and 5) has failed to demonstrate anti-nutritional effects when these compounds are fed at levels that are comparable to the inclusion of 30 % RSM in broiler diets. On the contrary, there is evidence that very low levels of these compounds may benefit the host through improved AME and apparent protein digestibility.

Despite the speculation that the simple phenolics (SNP and SA) are anti-nutritional factors, very little research has been done on this topic. In recent years, SA has been shown to be a strong antioxidant (Nowak et al., 1992; Wanasundara et al., 1996) and also to have antimicrobial activity *in vitro* (Nowak et al., 1992; Tesaki et al., 1998; Hua et al., 1999). These characteristics suggest that SA might have beneficial effects.

Three *in vivo* experiments were conducted to study the impact of SNP and SA on digestive tract bacteria. More specifically, the research investigated the effect of dietary SNP and SA on bacterial fermentation in the gastrointestinal tract (ileum and ceca) of chickens as indicated by volatile fatty acid production.

6.3 Materials and Methods

6.3.1 Animal and diet

Experiment 1. The experiment was based on a complete randomized design in which 120 day-old male broiler chickens (*Peterson* X *Hubbard*) were randomly assigned to ten treatments, with four replications per treatment and three birds per replication. Treatments consisted of a corn-soybean meal based diet as a control and the same diet supplemented with graded levels (0.15, 0.225, and 0.30 %) of SNP in purified (SNP bisulfate trihydrate) or semi-purified (ethanol extract concentrate) form (for source details, see Chapter 3.1). Three other treatments contained 15, 22.5 and 30 % RSM which resulted in SNP contents equivalent to the dietary levels of the purified and semi-purified treatments. The RSM in this research contained 1 % SNP as analyzed. Experimental details, including diet composition, were as described in Chapter 4.

Experiment 2. Eighty day-old commercial broiler cockerel chicks (*Peterson* X *Hubbard*) were fed five diets based on corn-soybean meal with one diet free of SA as the control, and another four diets containing graded levels of SA (0.05, 0.10, 0.15 and 0.20 %) which were equivalent to the SA profiles of the SNP moiety in diets containing 7.5, 15.0, 22.5, and 30.0 % RSM. SA was purchased from Sigma Chemical Co. (P.O. Box 14508 St. Louis, MO 63178 USA). Bird management and diet composition were as described in Chapter 5.

Experiment 3. To confirm the results of first SA trial (Experiment 2), this experiment was designed to repeat the VFA measurement by increasing the chick numbers within the replication. Ninety six male broiler chicks (*Peterson* X *Hubbard*) were randomly assigned to four treatments, with six birds in each replication and four replications for each treatment. A corn-soybean based diet served as a control, while another three diets were supplemented with graded levels of SA (0.025, 0.05 and 0.10 %). Bird management was as previously described in Chapter 5.

6.3.2 Measurement

Volatile fatty acid (VFA) measurement. Performance characteristics for birds in this research were reported in Chapter 4 and 5. For all three experiments, the status of bacterial populations in the ileum and ceca of experimental birds was assessed by examining VFA production. At 18 d of age, the birds were killed by cervical dislocation in Exp.1 and lethal injection with T-61®(Euthanasia solution) in Experiments 2 and 3, and the ileal and cecal digesta of three birds (Experiment 1 and 2) or six birds (Experiment 3) within each replication were collected in a well-sealed plastic centrifuge

tube and then frozen at –20°C. Ileal digesta were collected from the terminal ileum (distal half excluding the last 2 cm anterior from the ceca). Samples within a replication were pooled and mixed well prior to VFA measurement.

Analyses of VFA (acetic, propionic, isobutyric, butyric, isovaleric, and valeric acid) were conducted by Gas Chromatography (GC) (Varian Star 3400) using a Stabilwax®-DA column (0.25 mm ID, 0.25 μm df) (RESTEK Corporation, 110 Benner Circle, Bellefonte PA, 16823-8812 USA) and based on the procedure of Choct (1996) with minor modification which included the use of a pure volatile fatty acid (4-methyl valeric acid in 10% formic acid) that is not normally found in intestinal contents as an internal standard. A concentration of 2 mmol/L is acceptable for the internal standard in intestinal content samples. One ml of standard solution was added to 0.2 (cecal) or 0.5 (ileal) g of sample and vortexed until well-mixed. The mixture was then centrifuged at 28,000 g for 10 minutes and the supernatant pipetted into GC vials for analysis.

Microbial community assay. In Experiment 3, an assessment of the microbial community in cecal samples was conducted by Danisco Cultor Corporation, (FIN-02460, Kantvik, Finland). Samples were analyzed according to the method of Apajalahti et al. (1998). Cecal digesta samples from 2 birds out of 6 birds within each replication were pooled. Bacterial cells were isolated from the digesta by a five-cycle differential centrifugation process with sodium phosphate buffer. Bacterial cells were lyzed by enzymatic incubation with lysozyme followed by SDS incubation with mechanical agitation with glass beads. Samples were then sequentially extracted with CTAB (hexadecyltrimethyl ammonium bromide) and chloroform/isoamyl alcohol prior to alcohol precipitation of the nucleic acids. To obtain a profile of cecal digesta bacterial

communities based on by percentage-guanine-plus cytosine (%G+C) content, each DNA sample was subjected to cesium chloride-bisbenzimidazole gradient analysis and centrifuged in an ultracentrifuge. In this analysis, DNA quantitation was based on the UV absorbance at A_{280}. Determination of the %G+C content represented by each gradient fraction was accomplished by regression analysis of data obtained from gradients containing standard DNA samples of known %G+C composition (*Clostridium perfringens, Escherichia coli,* and *Micrococcus lysodeikticus*).

6.4 Statistical Analyses

All data collected were subjected to one-way Analysis of Variance (ANOVA) using the General Linear Model (GLM) procedure with a priori contrast option, as well as regression analysis (SAS® Institute, 1999). Differences were considered significant when $P<0.05$, unless stated otherwise.

6.5 Results

6.5.1 Experiment 1

Dietary treatment affected the level of ileal acetic acid (Tables 6.1 and 6.2). Regression of the control and treatments with three levels of SNP bisulfate trihydrate revealed a quadratic relationship between diet SNP and ileal acetic acid levels, with the lowest values found for the control and highest level of SNP treatment. Dietary treatment did not affect ileal propionic acid level. Regression analysis indicated a positive linear relationship between levels of dietary SNP bisulfate trihydrate and ileal butyric acid.

Table 6.1 The effect of sinapine source and level on the volatile fatty acid (VFA) content (μmol/g wet) in ileal and cecal digesta samples of broiler chickens (Experiment 1).

Treatment	Control	SNP bisulfate trihydrate			SNP ethanol extract			Rapeseed meal			SEM
SNP	0	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	0.15 %	0.225 %	0.30 %	
Ileal											
Acetic	9.9	11.6	12.9	8.1	11.6	10.0	10.4	10.7	11.9	8.7	0.34
Propionic	0.432	0.591	0.410	0.438	0.598	0.549	0.571	0.580	0.623	0.445	0.026
Butyric	0.015	0.090	0.097	0.117	0.087	0.058	0.050	0.047	0.142	0.099	0.012
Iso-Valeric	0.064	0.098	0.222	0.164	0.199	0.164	0.128	0.124	0.190	0.185	0.010
Total	10.4	12.4	13.7	8.9	12.5	10.8	11.2	11.5	12.9	9.4	0.36
Cecal											
Acetic	57.3	55.8	53.9	52.6	63.8	53.3	58.2	63.0	67.3	61.7	1.57
Propionic	5.42	6.74	4.45	6.41	5.78	4.46	4.23	5.30	4.60	4.56	0.29
Butyric	17.7	14.7	13.2	17.3	13.7	15.2	16.4	13.1	15.7	12.2	0.66
Iso-Butyric	0.288	0.280	0.288	0.498	0.353	0.213	0.218	0.323	0.315	0.300	0.025
Valeric	1.23	1.18	0.975	1.31	0.963	1.01	1.10	1.09	1.28	1.19	0.041
Iso-Valeric	0.618	0.598	0.503	0.683	0.535	0.485	0.608	0.623	0.593	0.563	0.024
Total	82.6	79.2	73.3	78.7	85.1	74.7	80.8	83.4	89.7	80.5	1.95

Table 6.2 One way analysis of variance (ANOVA) using GLM with a priori contrast and regression analysis of the volatile fatty acid (VFA) content of ileal and cecal digesta samples (Experiment 1).

VFAs	Ileal					Cecal						
	Acetic	Propio.	Butyric	Isovaler.	Total	Acetic	Propio.	Isobuty.	Butyric	Isovaler.	Valeric	Total
GLM	0.03*	ns	ns	0.001**	0.04*	ns	ns	ns	ns	ns	ns	ns
Planned contrast												
1 vs 2,3,4	ns	ns	0.06	0.002**	ns	ns	ns	ns	ns	ns	ns	ns
1 vs 5,6,7	ns	0.14	ns	0.001**	ns	ns	ns	ns	ns	ns	0.18	ns
1vs 8,9,10	ns	ns	0.08	0.001**	ns	ns	ns	ns	0.12	ns	ns	ns
Regression												
1, 2, 3, 4	ns	ns	0.04*	0.02*	ns	ns	ns	0.18	ns	ns	ns	ns
Linear	ns	ns	0.05*	0.02*	ns	ns	ns	0.18	ns	ns	ns	ns
Quadratic	0.01**	ns	ns	0.20	0.01**	ns	ns	ns	0.17	ns	ns	ns
1, 5, 6, 7	ns	ns	0.12	0.02*	ns	ns	ns	ns	ns	ns	ns	ns
Linear	ns	ns	0.09	0.001**	ns	ns	ns	ns	ns	ns	ns	ns
Quadratic	ns	ns	0.06	0.001**	ns	ns	ns	ns	ns	ns	0.19	ns
1, 8, 9, 10	ns	ns	0.14	0.001**	ns	0.18	ns	ns	0.11	ns	ns	ns
Linear	ns	ns	0.16	0.001**	ns	0.19	ns	ns	0.12	ns	ns	ns
Quadratic	0.17	0.15	ns	ns	0.18	ns	ns	ns	ns	ns	ns	ns

Propio. – propionic acid, Isovaler. – isovaleric acid, Isobuty. – isobutyric acid.

All treatments containing SNP were found to have a higher level of ileal iso-valeric acid with responses being linear for SNP bisulfate trihydrate, quadratic for ethanol extract, and linear for RSM. Total VFA changes followed the same pattern as acetic acid with a quadratic response associated with feeding SNP bisulfate trihydrate. Cecal VFA were not affected by dietary treatment.

6.5.2 Experiment 2

In the ileum, GLM analysis did not reveal a significant effect of dietary treatment but regression analysis supported a positive linear relationship between dietary SA and ileal levels of acetic, butyric and isovaleric acids, and total VFA (Table 6.3). Ileal propionic acid levels were unaffected by SA level. No valeric acid or isobutyric acid was detected in the ileum digesta of broiler chicks.

Inclusion of SA in the diet caused a significant quadratic reduction in the level of acetic acid and total VFA in cecal contents (Table 6.3). In general, the levels of acetic acid and total VFA were reduced by 15-20 % compared with control. Dietary treatment did not affect the level of propionic, butyric, valeric, isovaleric, and isobutyric acid in the cecal contents.

6.5.3 Experiment 3

Increasing levels of dietary SA resulted in a quadratic increase in the level of acetic acid and total VFA in the ileal digesta of broiler chickens (Table 6.4). There was no butyric, isobutyric or valeric acid found in ileal samples in this trial while the effect of diet on propionic acid and isovaleric acid was not significant. In contrast, cecal levels of

Table 6.3 The effect of dietary SA on the volatile fatty acid (VFA) content (μmol/g wet) of ileal and cecal digesta samples from broiler chickens (Experiment 2).

Treatment	Sinapic acid					SEM	GLM	Contrast	Regression	
VFA	0 %	0.05 %	0.10 %	0.15 %	0.20 %		(P)	1 vs 2-5	Linear	Quadratic
Ileal										
Acetic	9.07	9.12	9.32	12.01	11.26	0.50	ns	ns	0.04*	ns
Propionic	0.528	0.438	0.508	0.528	0.503	0.028	ns	ns	ns	ns
Butyric	0.090	0.098	0.093	0.113	0.130	0.006	0.13	ns	0.01**	ns
Iso-Valeric	0.121 ^{ab}	0.061 ^{b}	0.118 ^{ab}	0.151 ^{a}	0.157 ^{a}	0.011	0.03*	ns	0.03*	ns
Total	9.81	9.72	10.04	12.80	12.06	0.519	ns	ns	0.04*	ns
Cecal										
Acetic	70.4 ^{a}	52.0 ^{b}	51.2 ^{b}	52.1 ^{b}	53.2 ^{b}	2.26	0.01**	0.0006**	0.01**	0.01**
Propionic	3.29	4.14	3.20	4.00	4.96	0.30	ns	ns	0.14	ns
Butyric	14.7	15.6	16.4	17.7	15.2	0.59	ns	ns	ns	ns
Iso-Butyric	0.308	0.310	0.143	0.370	0.335	0.05	ns	ns	ns	ns
Valeric	0.94	0.79	0.85	1.22	1.17	0.08	ns	ns	0.12	ns
Iso-Valeric	0.583	0.580	0.443	0.675	0.548	0.04	ns	ns	ns	ns
Total	90.2	73.4	72.2	76.1	75.5	2.52	0.14	0.01**	0.11	0.06

$^{a, b}$ The values with different alphabets of superscript within each row are significantly different (P<0.05).

SEM – standard error of means.

Table 6.4 The effect of dietary SA on the volatile fatty acid (VFA) content (μmol/g wet) of ileal and cecal digesta samples for broiler chickens (Experiment 3).

Treatment	Sinapic acid				SEM	GLM	Contrast	Regression	
VFA	0 %	0.025 %	0.05 %	0.10 %		(P)	1 vs 2,3,4,5	Linear	Quadratic
Ileal									
Acetic	5.52 [b]	8.32 [a]	9.14 [a]	8.39 [a]	0.42	0.002**	0.0002**	0.001**	0.004**
Propionic	0.35	0.31	0.28	0.34	0.035	ns	ns	ns	ns
Iso-Valeric	0.058	0.055	0.013	0.055	0.009	ns	ns	ns	ns
Total	5.96 [b]	8.69 [a]	9.43 [a]	8.81 [a]	0.43	0.005**	0.0007**	0.002**	0.01**
Cecal									
Acetic	106.9 [a]	93.2 [a]	74.3 [b]	68.2 [b]	4.73	0.002**	0.001**	0.0001**	ns
Propionic	6.00	4.15	4.29	4.34	0.41	ns	ns	ns	ns
Butyric	18.1	19.9	16.3	16.7	1.00	ns	ns	ns	ns
Iso-Butyric	0.503	0.248	0.333	0.318	0.04	ns	0.04*	ns	ns
Valeric	1.60	1.44	1.28	1.06	0.09	0.14	0.08	0.02*	ns
Iso-Valeric	3.95	3.34	2.58	0.93	0.45	0.08	0.08	0.01**	ns
Total	137.0 [a]	122.3 [a]	99.1 [b]	91.6 [b]	5.74	0.004**	0.003**	0.0003**	ns

[a, b] The values with different alphabets of superscript within each row are significantly different ($P<0.05$).

SEM – standard error of means.

acetic acid and total VFA in cecal contents decreased in a linear fashion as the level of dietary SA increased. Levels of acetic acid and total VFA were reduced by 10-30 % depending on dietary SA level. Valeric and iso-valeric acids followed a similar trend as indicated by regression analysis while SA addition resulted in reduced isobutyric acid in comparison to the control as indicated by planned contrast analysis. Propionic and butyric acid were not affected by level of SA.

Microbial community analysis. Further microbial analysis in the cecal samples of Experiment 3, using the percentage-guanine-plus cytosine content in the DNA disclosed that dietary SA increased the relative abundance of bacteria in the %G+C 20-30 and 55-69 categories where the relative abundance values ranged from 1-5 % and 5-12 %, respectively, and decreased the amounts of microbes in the %G+C 40-54 region which had the highest relative abundance values ranging from 15-25 %. These effects were more significant as the dosage of SA given to the broilers was increased. Thus, it appeared that SA causes a shift in the relative proportion of cecal bacteria without changing the total bacterial numbers in the cecal microbial community (Figure 6.1).

6.6 Discussion

This is the first study to demonstrate that dietary RSM simple phenolics affect volatile fatty acid production (fermentation) and the microbial ecology in the gut of broiler chickens. This is in contrast to previous phenolic research which investigated the impact of gut microbes on the metabolism of simple phenolic acids (Scheline, 1966, 1968; Griffiths, 1969; Jung et al., 1983a,b). It was found that the major simple phenolics in RSM, SNP, increased ileal microbial fermentation but did not affect the cecal

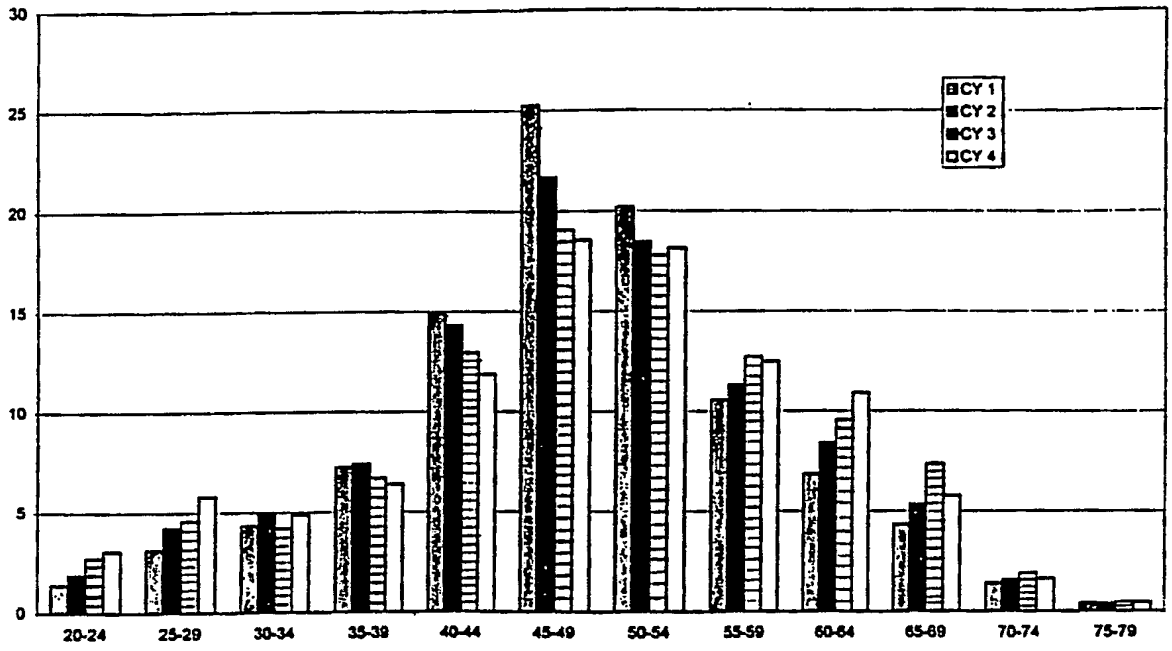


Figure 6.1 Percentage G+C profiles of cecal microbial community and relative abundance of bacteria in different ranges of %G+C (CY 1: control, CY 2: SA 0.025 %, CY 3: SA 0.05 %, CY 4: SA 0.10 %).

fermentation in broiler chickens. In comparison, dietary SA consistently increased the short chain VFA level in the ileum and decreased VFA levels in the ceca. The predominant reduction of VFA in the ceca suggests that SA might have antibacterial activity *in vivo*.

The effect of dietary SNP bisulfate trihydrate on VFA production in the ileum of broiler chickens was due to a quadratic increase in acetic acid and linear increases in isovaleric and butryic acids. The total ileal VFA response to SA was similar to acetic acid because of the predominance of this VFA in ileal digesta. The reason for this effect is unclear but could be either a direct effect of SNP or an indirect effect of SA, choline or other metabolites produced as a result of microbial hydrolysis of SNP. As shown earlier in the thesis (Chapter 4), approximately 30-40 % of dietary SNP has disappeared and is presumably hydrolyzed by the ileum. SA may be a logical candidate for this effect as the results of Experiments 2 and 3 of this research show a similar response in ileal VFA in response to dietary SA. It is also difficult to judge the biological or production significance of this change without knowing more about the microbial changes that are associated with changes in VFA production. If absorbed, the increase in VFA production may contribute positive energy to the host and/or VFAs could change the pH of small intestinal digesta in a manner that improves nutrient utilization. The lower pH potentially encourages the establishment of a beneficial microflora in the upper intestines while at the same time reducing the growth rate of pathogenic bacteria in turn the competition on nutrients (Ratcliff, 2000). The fact that diet AME increased in the same quadratic pattern as VFA content to increased dietary SNP bisulfate trihydrate supports this possibility (Chapter 4). Alternately, increased numbers of bacteria and/or changes in microbial ecology have the potential to adversely affect the host. The data from this research are inconclusive but the lack of a negative effect on production

and a positive effect on nutrient utilization would suggest no major effect or a positive response. SNP did not impact cecal VFA which appears to be in agreement with Nowak et al. (1992) who found that SNP demonstrated weak antibacterial activity *in vitro*. The fact that dietary SNP reduced empty cecal weight seems somewhat contradictory to this finding. Since the hind gut of chickens is a major site of microbial SNP hydrolysis, it is of interest that released SA did not affect cecal fermentation as found when SA was included in the diet.

In two trials, dietary SA consistently caused a linear increase in ileal VFAs and a dramatic decrease in cecal VFA. As noted above for SNP, the significance of the SA effect in the small intestine is unknown and requires further study. However, the increase in ileum is in contrast to the decrease in cecal VFA content. This may be related to known differences in bacterial populations in the small intestine and ceca. As an example, the small intestine is colonized with more aerobic bacteria and anaerobes are predominant in the ceca (Mead, 1997). A different response by these organisms to SA could explain the apparent discrepancy. The preliminary finding that SA causes changes in the proportion of organisms in the ceca rather than total numbers is supportive of this possibility (see below). SA has also been shown to affect bacteria *in vitro*. Tesaki et al. (1998) demonstrated that SA from mustard possessed antibacterial activity against *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus aureus*.

The *in vitro* demonstration of SA antibacterial action involved direct interaction between SA and bacteria. In the current *in vivo* situation, SA is apparently almost entirely absorbed by the terminal ileum so may not access the ceca. The lack of SA in the ceca supports this idea. If this is the case, SA would have to mediate its *in vivo* effect by a more indirect mechanism. This could be due to a direct effect of a microbial

metabolite of SA that enters the ceca and alters the microbial ecology or via a systemic effect mediated by absorbed SA. Further research on SA metabolism is required to establish the metabolic fate of SA and the mechanism whereby it affects hind gut fermentation.

The strong inhibitive impact of SA on total cecal VFA production may be nutritionally beneficial to the animal host. Studies on soluble fiber and non-starch polysaccharides from various feed ingredients indicate increased fermentation in the hind gut produces energy from undigestible fibrous feed ingredients which may be positive to energy utilization, but growth-depressing effects are often observed. This is due to increased microbial growth in the hind gut, producing bacterial catabolites, such as ammonia, amine, indoles, hydrogen sulfide, secondary bile acids, and short chain fatty acids. Bacterial catabolites may be detrimental to animal health and have a negative effect on gut function (Slominski et al., 1994; Choct et al., 1996; Mead, 1997; Langhout et al., 2000). Large ceca are often observed in birds when fed highly fermentative undigestible feed ingredients. These points are also supported by some models using purified or semi-purified diets, either in conventional or germ-free chickens (Iji et al., 1998; Langhout et al., 2000). Therefore, reduced fermentation in the lower gut is generally considered to be beneficial.

Further microbial community assay found that the anti-fermentative effect of SA in ceca may be mediated via the modulation on some specific microbial species. The shift in the relative proportion of cecal bacteria without changing the total bacterial numbers, suggests the decreased VFA production may be due to effect of SA on selective bacterial species. Dietary SA increased the relative abundance of bacteria in the ranges

of %G+C 20-30 and 55-69 and decreased the relative abundance of microbes in the range of %G+C 40-54. The shift was more significant as the dosage of SA given to the broilers was increased. These data suggests that dietary SA increased the relative abundance of a small portion of *Clostridium* in the range from 20-30, and *Bifidobacterium* and *Propionibacterium* from 55-69. In comparison, SA decreased the relative abundance of *Escherichia*, *Salmonella*, partial Bacteroides, *Eubacterium* and *Lactobacillus* in the range from 40-54 which are the most abundant bacterial genera present in the GI tract of the chicken (Figure 6.2, Apajalahti et al., 1998). These changes, in general, supported an increase in the relative abundance of bacteria generally considered beneficial, such as *Bifidobacterium* and *Propionibacterium*, and a decrease for potentially undesirable bacteria, such as *Escherichia* and *Salmonella.* Thus this shift might be microbiologically and in turn nutritionally beneficial. *E. coli* and *Salmonella* are the most common pathogens in the animal production and probiotics such as microbial agents derived from *Bifidobacterium* and *Lactobacillus* are frequently used as animal health promotants. Therefore, SA may increase animal productivity by increased host health status. From this point, SA might be considered a growth promotant and an alternative to antibiotic, and prebiotics or probiotics. Antibacterial properties of SA were detected for some selected bacterial strains, such as *E. coli* and *S. enteritidis* (Tesaki et al., 1998), which is consistent with current *in vivo* study. However, the exact reason for this shift must be complicated due to the complexity of gut environment, diversity of hind gut microbes and our limited knowledge on the interrelations among dietary fiber components, gut microbes, and dietary SA when RSM was fed. The mechanism of whether SA inhibits acetic acid production through inhibition of specific bacteria or a shift in the microbial

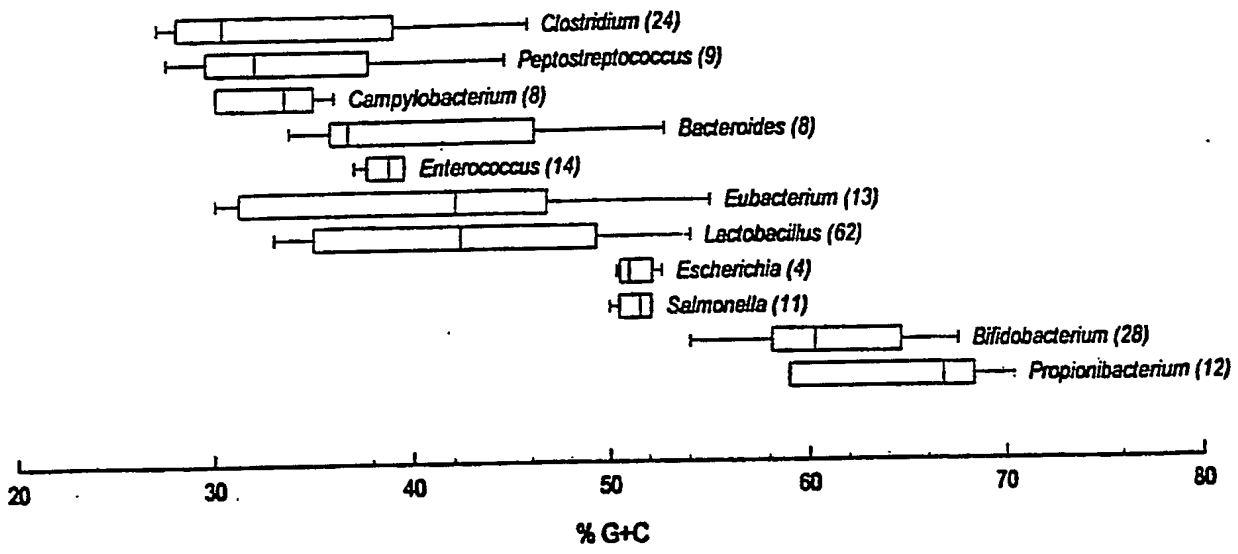


Figure 6.2 Ranges of percent G+C content in bacterial genera present in the GI tract of the chicken. Boxes indicate ranges, which accommodate 80 % of the species within a given genus, and the vertical line in each box is the median of that genus. The values in parentheses show the number of species included in the survey. The figure is based on the literature data (Apajalahti et al., 1998).

community profile in the hind gut of broiler chickens remains unknown and needs to be further investigated.

Up to date, there is no other research indicating that simple phenolic compounds may affect gut microorganisms in broiler chickens, although indirect evidence on phenol metabolism implicates the involvement of gut microflora in other animal species. The unique study in the mechanism of cinnamic acid absorption in rats (Wolffram, 1995) indicated radioactively labeled cinnamic acid was absorbed in the jejunum of rat, and short chain fatty acids (acetic, propionic and butyric acid) significantly inhibited Na^+-dependent uptake of radioactively labeled cinnamic acid. This demonstrated the substantial interaction between phenolic acids and volatile fatty acids which involves the gut microbes.

This is the first research disclosing the antibacterial activity of dietary SA *in vivo*. Previous study on the metabolism of SA (Chapter 5) demonstrated the predominant absorption of SA occurred prior to the terminal ileum, suggesting the participation of SA in the blood systematic circulation. No SA found in the ileum or ceca of broiler chicks also supports the substantial absorption in the upper gut. However, after absorption, how the SA is metabolized and is involved in the interaction with other nutrients in the circulation system or in the tissues or organs, and how SA reduces the cecal fermentation through the modulation on hind gut bacteria, are apparently not clear.

The improved nutrient utilization with SA especially at low levels and the reduced fermentation pattern in the hind gut of broiler chicks, suggests the possibility of SA used as a growth promotant in animal feeding. The two SA trials were conducted at a clean experimental environment so that a growth-promotion effect was not expected in the

current research. However, SA at the additional low level of 0.025 % in Experiment 3 stimulated the feed intake along with enhanced nutrient utilization and performance, which suggests the idea that SA might be a strong candidate as growth promotant. In commercial animal production conditions, SA may be used as a natural alternative to antibiotics, and more activity is expected on the inhibition of sub-clinical symptoms, reduction of microbial metabolites, reduction of microbial competition for nutrients, and with enhanced uptake and utilization of nutrient.

6.7 Conclusion

SNP bisulfate trihydrate derived from RSM had no effect on the short chain VFA production in the ceca of broiler chickens, but did cause a linear increase in isovaleric and butyric acids and a quadratic response to acetic acid in the ileum. Therefore, it appears that SNP does not have anti-bacterial activity as indicated by VFA production. Data from this research demonstrated the strong inhibitory effect of dietary SA on short chain VFA production in the ceca of broiler chicks, especially acetic acid and total VFA production, suggesting the SA in RSM might have antibacterial activity *in vivo* in broiler chickens. This is likely nutritionally beneficial to animal host. However, the mechanism of dietary SA on the inhibition of VFA production in the hind gut or the alteration of microbial community profile needs to be investigated. Further study is justified to determine if SA could act as a natural growth promotant to improve the performance of non-ruminant animals production.

7. ENZYME SCREENING TESTS FOR BREAKDOWN OF SINAPINE IN VITRO AND HYDROLYSIS OF SINAPINE IN RAPESEED MEAL WITH FERULIC ACID ESTERASE

7.1 Abstract

Sinapine (SNP) is an ester of sinapic acid (SA) and choline which is found in significant amounts in rapeseed and rapeseed meal. It is not broken down by endogenous intestinal enzymes in non-ruminant species but can be hydrolyzed into its basic components by intestinal microorganisms. In the chicken, this hydrolysis can occur before the ileum of the small intestine but most is apparently hydrolyzed after this point. As a consequence, released choline is further metabolized to trimethylamine which can cause fishy flavored eggs in some strains of laying hens. The objective of this research is to find enzymes that are capable of hydrolyzing SNP *in vitro*, potentially making choline more available for absorption in the upper section of the small intestine and taking advantage of potential beneficial effects of SA. A total of four enzymes were chosen based on esterase activity and tested *in vitro* using pure sinapine bisulfate as a substrate. Tannase, which is a tannin acylhydrolase, had only low hydrolytic activity and possibly contained other non-specific enzyme activities. Tyrosinase, a monophenol oxidase, effectively broke down SNP, but also transformed SA to other oxidized products with unknown toxicological properties. At 30-40°C, and pH 6.0, the disappearance of SNP was determined as 80 % after 60 minutes incubation. A third enzyme, phenolase (diphenol hydrolase) was not capable of hydrolyzing SNP. Ferulic acid esterase (FAE), a

subclass of carboxylesterase, demonstrated strong hydrolytic activity towards SNP in both pure form and as present in RSM, producing SA and choline. At 50-60°C and pH 4.0-6.0, 100% of sinapine bisulfate was hydrolyzed after 15 minutes incubation. At 50°C and pH 4.0 (citric acid buffer), the SNP content in commercial rapeseed meal was reduced by 85 % after 20 minutes treatment. This enzyme possesses a broad optimal temperature and pH range, and is a strong candidate for efficiently and specifically hydrolyzing SNP in RSM.

(*Key words*: enzymes, sinapine, sinapic acid, choline, rapeseed/canola meal)

7.2 Introduction

Rapeseed meal contains the simple phenolic compounds, sinapine and sinapic acid, which are usually considered to have negative effects in animal production and food processing. Their negative effects include reduced diet palatability, fishy egg taint problem in some strains of laying hens, protein binding and other deleterious organoleptic properties of using canola or rapeseed protein products (Josefsson et al., 1976; Sosulski, 1979; Fenwick et al., 1984; Kozlowska et al., 1990; Shahidi et al., 1992, 1995). In addition, the choline availability in rapeseed meal (RSM) is much lower than in soybean meal (SBM), although RSM is three times as rich in total choline as SBM (Emmert et al., 1997). Since the majority of RSM choline is found as a portion of SNP, it is possible that this fraction is less available than free or other forms of choline. Choline availability may also be affected by other components of RSM. As a consequence of these perceived negative effects, research has been and is being conducted to reduce or eliminate the SNP content in rapeseed meal using plant breeding, physical or chemical treatment and enzymatic processing (McGregor et al., 1983; Diosady et al., 1986; Shahidi et al., 1989; Lacki et al., 1997, 1998).

Plant breeding may be the best and most efficient way to decrease the SNP content in the seed with at least one current research project attempting to block the synthesis of SNP during seed development using molecular biology (Selvaraj et al., 2000). However, to date, no rapeseed cultivars low in SNP or SA content have been licensed. Although several physical and chemical methods have been proposed for RSM processing, none could effectively and efficiently remove the SNP content of the meal.

Dehulling does not decrease SNP content since SNP mainly exists in cotyledons. Dry extrusion and other heat treatments have been unable to substantially alter the SNP content of RSM. Chemical treatments offer some potential to reduce SNP content, such as the use of sodium carbonate, limestone, ammonia with steam, or ammonia-methanol-water treatment, and better results are obtained when combined with physical heating treatment. However, these treatments can also cause a significant nutrient loss during processing. For example, Na_2CO_3 treatment reduced the meal content of SNP by 20-40% but reduced available lysine by 15-20 % (Bell, 1981). During RS crushing, high temperature employed in desolventizing, in combination with high moisture treatment during toasting , may darken the color of meal due to the Maillard reaction (Newkirk and Classen, 2001), and it is also possible that it results in oxidation of phenolic compounds. However, the products derived from this process have never been evaluated for SNP content and may decrease the quality of the final meal.

Recently Lacki and Duvnjak (1996, 1997, 1998) proposed an enzymatic method to decrease the SNP content of RSM. SNP in RSM was transformed by an enzyme secreted by a white rot fungus *Trametes versicolor*, which is a polyphenol oxidase. When RSM was treated with this enzyme during the crushing process, the phenolic content was decreased by 90 % under optimal temperature and pH conditions (Lacki et al., 1996). The enzymatic process could also be carried out in the presence of hexane and SNP was decreased by 97 % (Lacki et al., 1998). However, from a biochemical point, the products of SNP breakdown are not only SA and choline but most likely quinone(s) or quinone derivatives derived from the action of the polyphenol oxidase (oxidation) treatment on SNP or SA. These compounds are very biologically active compounds and

more toxic than phenols. Thus, the breakdown of phenolic compounds in RSM by polyphenol oxidase needs to be questioned and to date has not been approved.

The results of research using purified SNP or SA presented in this thesis (Chapter 4, 5, 6) suggest that these phenolics do not have toxic effects on broiler chickens. In contrast, beneficial effects on nutrient utilization were associated with feeding low levels of these phenolics. Both SNP and SA improved the nutrient utilization and SA may also have antibacterial activity *in vivo*. A low level of SA stimulated the feed intake and performance. Thus, the hydrolysis of SNP into SA and choline may prove to be beneficial from both the SA and choline release standpoints. It can be speculated that enzymatic hydrolysis of RSM SNP prior to feeding would result in rapid absorption of choline in the upper small intestine rather than being transformed by bacteria in the lower gut to compounds such as trimethylamine. If this were the case, RSM could provide a portion or the animal's entire choline requirement depending on level of meal inclusion in the diet.

Based on the premise that SNP hydrolysis to SA and choline would improve the nutritional value of RSM, the objective of this research was to study the potential of several selected enzyme sources to hydrolyze SNP *in vitro* and not be active towards the resulting SA in terms of further modification to quinones or quinone derivatives.

7.3 Materials and Methods

Four enzymes were chosen based on their published enzyme activity and their potential to hydrolyze SNP *in vitro* (Table 7.1). Tannase (tannin acylhydrolase) derived from *Aspergillus oryzae* possesses activity towards the ester bonds of tannins, and

Table 7.1 Enzymes selected to test for their ability to breakdown sinapine.

Enzyme	Source	Primary enzyme activity	Substrate	Optimal temperature	Optimal pH
Tannase	*Aspergillus oryzae*	Tannin acylhydrolase	Tannins	30-40 °C	5.0-6.0
Tyrosinase	Mushroom	Tyrosinase, monophenol oxidase	Tyrosine	25 °C	6-7
Phenolase	Potato	Diphenol oxidase, phenolase	Chlorogenic acid	30 °C	7.5
Ferulic acid esterase	*Aspergillus*	Ferulic acid esterase, carboxylic ester hydrolase	Methyl ferulate	30-40 °C	6.0

Table 7.4 The breakdown of sinapine bisulfate trihydrate by ferulic acid esterase treatment *in vitro* under different temperature and pH conditions.[1]

Time (min) / Condition	0	0.5	1	2	4	6	8	10	14	18	22	26	30	Reduction by %
25 °C, pH 4.0	30	-	-	-	-	9.4	-	9.7	10.3	-	7.7	3.9	3.7	87.6
30 °C, pH 4.0	59.7	49.6	-	46.1	44.1	43.2	34.2	36.0	33	32.7	30.5	27.1	19.8	66.8
40 °C, pH 4.0	30	18.8	-	5.5	5.0	3.4	6.2	5.0	3.3	1.73	1.68	1.66	1.22	95.9
50 °C, pH 4.0	27.7	14.1	10.2	2.7	2.9	1.8	1.1	0	0	0	0	0	0	100
60 °C, pH 4.0	27.7	11.6	10.1	5.4	4.4	1.6	1.1	1.0	0	0	0	0	0	100
50 °C, pH 5.0	37.2	18.9	15.4	11.3	5.9	3.1	2.0	1.7	1.1	0	0	0	0	100
50 °C, pH 6.0	41.0	6.6	3.3	2.9	1.9	1.7	1.4	1.2	0	0	0	0	0	100

[1] The original liquid enzyme was diluted 10 times. The sinapine standard was dissolved in either redistilled water or citric acid buffer at different pH range. The injection volume of ferulic acid esterase was 10 µl. The reaction volume was 1 ml. 10 µl sample solution was applied to the HPLC column for analysis after enzyme treatment. High temperature was used to terminate the reaction, each test tube was placed in a boiling waterbath for 2 min prior to HPLC analysis.

Analysis of SNP and SA (tannase, tyrosinase, and phenolase tests) was based on the UV detection methods (Chapter 3.2). The maximum absorbances of SNP and SA were measured under the wavelength at 328 nm and 310 nm, respectively. HPLC based on the fluorescence detection method (EX: SNP – 318 nm, SA – 280 nm; EM: SNP – 465 nm, SA – 428 nm) and a reversed-phase column were used for the ferulic acid esterase test. For sample preparation of RSM see Chapter 3.2.2.2.

7.4 Results

Tannase was able to hydrolyze SNP into sinapic acid and choline (Table 7.2), but the reaction time was relatively long. As an example, at optimum conditions of 30°C, and pH 5.5, tannase hydrolyzed 86.2 % of SNP after 120 minutes incubation. Under these conditions, this enzyme source was only marginally able to further modify SA (Table 7.2). This minor activity may be either attributable to the acyl hydrolase activity or other enzyme activities present in this relatively impure enzyme source. However, there was no colored product formed after the reaction. The tannase was found to not be very stable to low temperature storage (4°C) and consequently assay results were not very repeatable.

Tyrosinase was more effective in hydrolyzing SNP under broader temperature and pH ranges compared with tannase. At 30-40 °C and pH 6.0, the disappearance of SNP was determined as 80% after 60 minutes incubation (Table 7.3). This enzyme is a phenol oxidase and therefore it has the ability to also breakdown SA, although this reaction was more sensitive to pH change (Table 7.3). It was observed that colored substances were

thereby hydrolyzes tannins into gallic acid. Since it is a crude enzyme preparation, it may also have other enzyme activities. This enzyme is used in the beverage industry such as for tea production to prevent haze formation. The crude enzyme product was provided by Finnfeed International Ltd. (Market House, Box 777, Ailesbury Court/High Street, Marlborough, Wiltshire, SN8 1AA, United Kingdom). Tyrosinase (from mushroom), purchased from Sigma Chemical Co.(P.O. Box 14508 St. Louis, MO 63178 USA), is classified as a monophenol oxidoreductase. Substrates are aromatic and phenolic compounds, and an example reaction is the conversion of tyrosine to dopaquinone. Phenolase (from potato) is a diphenol hydrolase using chlorogenic acid as substrate. It was purchased from Sigma Chemical Co. as a lyophilized powder. Ferulic acid esterase, obtained from Finnfeed International Ltd. is a carboxylesterase which is able to release ferulic acid from a range of esterified substrates including methyl ferulate and feruloylated oligosaccharides isolated from digested plant cell walls.

The substrates used in assays were SNP bisulfate trihydrate derived from RSM (See Chaper 3) with a purity greater than 96.3 %, SA which was purchased from Sigma Chemical Co. (98.0 %, titration), and a commercial RSM sample.

All enzymes were dissolved in either redistilled water or citric acid buffer to modify pH. A waterbath was used for temperature control. Substrate solutions were made in either redistilled water or citric acid buffer with HCI and KOH as acid-base modifier whenever necessary. Depending on the enzyme tested, the assay temperature ranged from 25 to 60°C and pH from 4 to 6. The reaction time varied from 15 to 120 minutes.

Table 7.2 Breakdown of SNP and SA by tannase *in vitro* as indicated by the reduction in the absorbance of a standard solution as measured by spectrometer.

Time (min) / Condition	0	15	30	45	60	75	90	105	120	Reduction[1]
SNP 20 °C, pH 4.5	0.238	0.189	0.171	0.167	0.183	-	-	-	-	23.1
SNP 20 °C, pH 5.5	0.291	0.257	0.243	0.222	0.199	0.181	0.176	0.149	0.139	52.2
SNP 30 °C, pH 4.5	0.232	0.208	0.206	0.201	0.198	0.193	0.195	0.181	0.177	23.7
SNP 30 °C, pH 5.5	0.247	0.192	0.172	0.142	0.109	0.092	0.069	0.051	0.034	86.2
SNP 40 °C, pH 4.5	0.230	0.207	0.207	0.211	0.201	-	-	-	-	12.6
SNP 40 °C, pH 5.5	0.284	0.249	0.238	0.216	0.206	0.198	0.196	0.192	0.177	37.7
SNP 50 °C, pH 5.5	0.290	0.235	0.236	0.243	0.232	0.244	0.237	0.221	0.235	19.0
SA 20 °C, pH 5.5	0.356	0.350	0.349	0.346	0.343	0.343	0.339	0.343	0.341	4.2
SA 30 °C, pH 4.5	0.426	0.420	0.412	0.411	0.407	0.402	0.399	0.402	0.400	6.1
SA 30 °C, pH 5.5	0.377	0.368	0.363	0.359	0.356	0.353	0.335	0.332	0.330	12.5

[1] Percent reduction after 120 minutes.

Table 7.3 The breakdown of SNP and SA by tyrosinase *in vitro* as indicated by the reduction in the absorbance of a standard solution as measured by spectrometer.

Time (min) / Condition	0	15	30	45	60	Reduction [1]
SNP 30 °C, pH 6.0	1.035	0.415	0.186	0.168	0.174	83.2
SNP 40 °C, pH 6.0	1.031	0.223	0.199	0.205	0.210	79.6
SNP 50 °C, pH 4.5	1.111	0.489	0.263	0.243	0.260	76.6
SNP 50 °C, pH 6.0	1.024	0.259	0.222	0.216	0.220	78.5
SNP 60 °C, pH 4.5	1.077	0.313	0.276	0.275	0.271	74.8
SA 30 °C, pH 6.0	0.440	0.401	0.405	0.426	0.467	-6.1
SA 40 °C, pH 6.0	0.428	0.412	0.442	0.465	0.474	-10.7
SA 50 °C, pH 4.5	0.608	0.255	0.235	0.254	0.260	57.2
SA 50 °C, pH 6.0	0.429	0.410	0.421	0.414	0.410	4.4
SA 60 °C, pH 4.5	0.639	0.263	0.284	0.288	0.290	54.6

[1] Percent reduction after 60 minutes.

formed in the end solution after enzymatic treatment. However, the colored substances were not identified in this assay. Phenolase did not demonstrate any activity towards SNP as indicated by a failure to alter the absorbance of the reaction media (data not shown).

Using HPLC for the quantification of SNP and SA, ferulic acid esterase was demonstrated to possess a strong ester hydrolyase activity in the hydrolysis of SNP. Ferulic acid esterase demonstrated effective hydrolysis of SNP *in vitro* under broad ranges of temperature and pH. At 50-60°C and pH 4.0-6.0 using either redistilled water or citric acid as a buffer system, 100 % of the SNP was hydrolyzed after 15 minutes incubation. Substrate concentration, enzyme dosage, incubation volume, reaction time, temperature and pH conditions were tested. The hydrolysis of SNP under different conditions is shown in Table 7.4. SA was identified as a hydrolysis product and it was confirmed that there was no further breakdown of SA (Table 7.5). These results demonstrated that this enzyme specifically hydrolyzes the ester bond within the SNP molecule.

The ferulic acid estease reaction was terminated by high temperature treatment for 2 minutes using a boiling waterbath instead of strong acid because the reaction was found to be reversible under acidic conditions (Table 7.6). With the addition of increasing volumes of 6M HCI to the reaction, the level of SNP in the test tube was increased. However, if 6M HCI or concentrated acetic acid was added into the substrate solution before the reaction, it would terminate the reaction immediately, possibly by inactivating the enzyme through degeneration of the protein.

Table 7.5 The *in vitro* hydrolysis of sinapine bisulfate trihydrate and formation of sinapic acid by ferulic acid esterase at 50 °C and pH 5.0 (citric acid buffer) [1,2].

Time (min)	SNP (μg/ml) concentration	SNP reduction by %	SA formation (μg/ml)
0	37.2	-	0
0.5	18.9	49.2	36.2
1	15.4	58.6	35.8
2	11.3	69.5	36.5
4	5.9	84.2	37.1
6	3.1	91.7	36.4
8	2.0	94.5	37.4
10	1.7	95.3	38.6

[1] The SNP and SA assay was conducted using HPLC. The injection volume of ferulic acid esterase was 10 μl. The reaction volume was 1 ml. 10 μl sample solution was applied to the HPLC column for analysis after enzyme treatment. High temperature was used to terminate the reaction, each test tube was placed in a boiling waterbath for 2 min prior to HPLC analysis.

[2] Every sample was treated in duplicate.

Table 7.6 The effect of acid addition on the hydrolysis of sinapine bisulfate trihydrate at room temperature 25 °C and pH 4.0. *

Reaction time (min)	Acid addition (µl)	SNP concentration (µg/ml)	SNP Reduction by %
0	-	30.0	-
6	na[1]	7.8	74.2
6	10 a[2] (HCl)[3]	14.8	50.7
6	10 b[4] (HCl)	40.1	-33.6
0	-	30.0	-
5	na	8.8	70.7
5	50 a (HCl)	17.9	40.3
5	50 b (HCl)	41.2	-37.3
0	-	29.7	-
5	na	13.9	53.2
5	200 a (HCl)	31.3	-5.4
5	200 b (Ace)[5]	31.5	-6.1

* The SNP assay was conducted using HPLC. The injection volume of ferulic acid esterase was 10 µl. The reaction volume was 1 ml. 10 µl sample solution was applied to the HPLC column for analysis after enzyme treatment.

[1] na – not applicable.

[2] a - HCl was added after the reaction.

[3] HCl – 6M HCl.

[4] b –HCl was added before the reaction.

[5] Ace – concentrated acetic acid.

The ferulic acid esterase was also used to hydrolyze SNP in a commercial RSM sample. At 50°C and pH 4.0 (citric acid buffer), the SNP content in commercial canola meal (normal particles without grinding) was reduced by 85 % after 20 minutes treatment (Table 7.7). Increasing the enzyme volume (40 or 80 µl) or reaction time to 200 minutes did not result in further breakdown on SNP, suggesting the enzyme at dosage 20 µl was already saturated by the substrate and appropriate for this enzyme assay (data not shown). The hydrolysis of SNP by FAE both in standard stock solution (50 °C, pH 5.0), and in commercial RSM sample (50 °C, pH 4.0) are shown in Figures 7.1 and 7.2.

7.5 Discussion

The successful hydrolysis of SNP *in vitro* requires an enzyme capable of hydrolyzing the ester bond between SA and choline. Of the four enzyme tested, tannase, tyrosinase and ferulic acid esterase were capable of breaking the SNP ester bond. Tannase had a mild hydrolase activity in the hydrolysis of SNP, and a minor activity in the modification of SA due to the impurity of this enzyme products. However, no colored substances were formed after the enzymatic treatment, suggesting the major activity of this enzyme preparation is acyl hydrolase.

The fact that tyrosinase could break down both SNP and SA, demonstrates that it possesses phenol oxidase activity. Due to the classification of tyrosinase as a monophenol monooxygenase, the enzymatic products of SA breakdown were probably quinones or their derivatives. This idea is supported by the formation of colored substances during the assay. Therefore, this enzymatic breakdown pathway of SNP may

Table 7.7 The breakdown of sinapine (SNP) in commercial canola meal by ferulic acid esterase (FAE) at 50 °C and pH 4.0 (citric acid as buffer).[1,2]

Time (min)	Concentration (μg/ml)	Average SNP content (%)	% Error	SNP Reduction by %
0	29.99	0.585	10.9	-
0	32.60			
5	9.24	0.176	22.2	69.9
5	11.11			
10	6.71	0.133	7.7	77.2
10	7.36			
20	5.65	0.090	23.5	84.6
20	4.44			

[1] Every meal sample was treated in duplicate. The % Error is the percentage error between the duplicates.

[2] The assay used 20 μl of ferulic acid esterase and 2 ml of stock solution, with the injection volume at 10 μl.

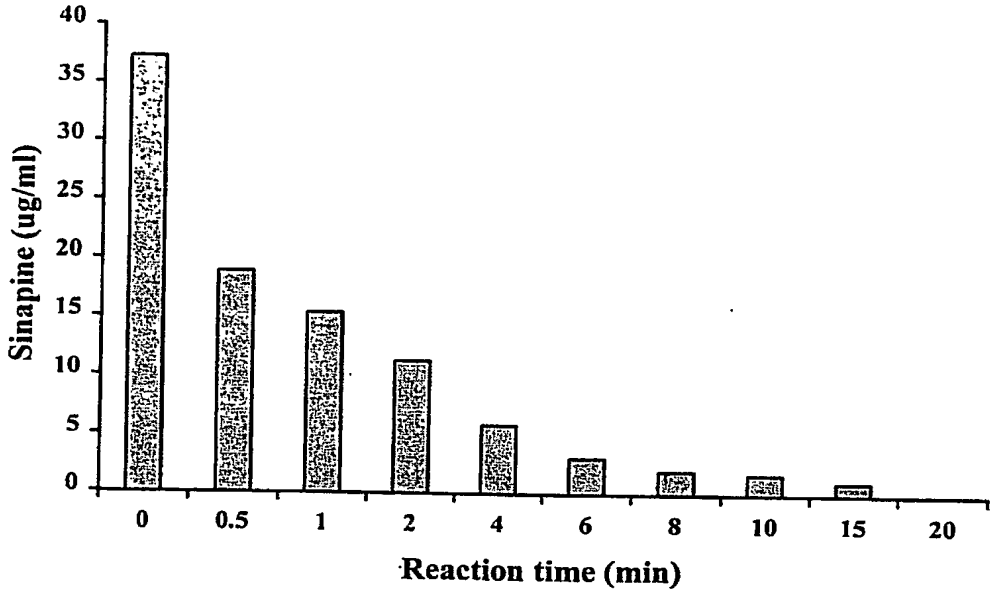


Figure 7.1 The hydrolysis of sinapine bisulfate trihydrate by ferulic acid esterase (FAE) in the standard stock solution at 50 °C and pH 5.0 as analyzed by HPLC.

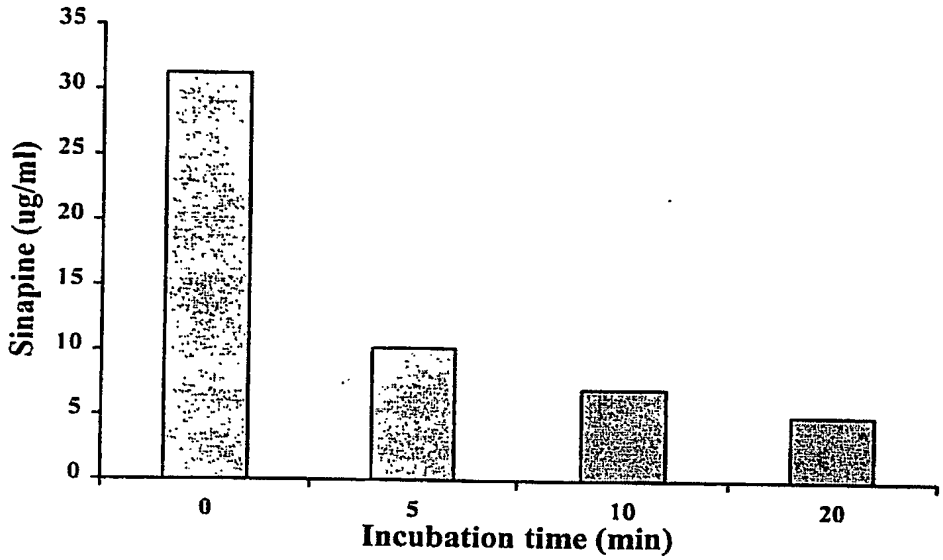


Figure 7.2 The hydrolysis of sinapine in commercial rapeseed meal by ferulic acid esterase (FAE) at 50 °C and pH 4.0 as analyzed by HPLC.

not be suitable for application in RSM processing. To date, the enzymatic breakdown of SNP in rapeseed meal using a polyphenol oxidase (Lacki et al., 1996, 1997, 1998) has not been used commercially in Canada. Because of the potential negative effects of SNP or SA breakdown products by (poly)phenol oxidase, another requirement of an industrially acceptable enzyme source is that it does not demonstrate activity against SA. Only the ferulic acid esterase met this requirement.

Ferulic acid esterase successfully hydrolyzed SNP in RSM. This is possible since this enzyme is an ester hydrolase and SNP has an ester bond which is similar to ferulic acid linkage in feed ingredients. In addition, ferulic acid is the previous precursor for SA synthesis so is chemically quite similar. This enzyme presented strong hydrolytic activity under a broad temperature and pH ranges *in vitro* and the failure of FAE to hydrolyze SA demonstrated the specific carboxylic ester hydrolase activity of this enzyme. In addition, the optimal pH range of this enzyme is near 5, which is close to the natural pH of RSM and not far from the physiological pH of animals. Thus, FAE could be utilized in the hydrolysis of SNP during meal processing or possibly added as a feed additive into the animal diet.

The advantages of SA and choline in animal nutrition have already been demonstrated in previous chapters (Chapters 4, 5, and 6). The hydrolysis of SNP into SA and choline during processing or in the upper small intestine proved to be beneficial. Releasing choline from SNP in the upper gut may increase the choline absorption and availability and reduce the potential for modification to trimethylamine by gut bacteria. SA was demonstrated to improve the nutrient utilization and may have strong antibacterial activity thereby producing a growth-promotion effect. Thus, this is

particularly important in selecting an enzyme that is capable of hydrolysis of SNP but does not affect SA.

HPLC analysis indicated (Table 7.5), interestingly, the considerable larger amount of SA detected in the products than calculated value, suggested the quantification of SNP must consider the pH conditions. It was disclosed that the spectral properties of SNP are closely correlated with the pH conditions (Chapter 3.3). Under the acid condition, possibly more SNP exists in the intact form and easily be detected. The impact of acid addition on the enzymatic hydrolysis of SNP (Table 7.6) may be such a reflection of pH effect on SNP spectral property. Higher SNP content was found in the acidified SNP standard stock solution than that without acidification. This is consistent with the previous study of the effect of pH on the spectral properties of sinapine bisulfate trihydrate standard solution. The fact that the yield of SA released from SNP hydrolysis did not change much with time (Table 7.5) is contradictory to the reduction of SNP in the stock solution. The reason is not apparent since it is not known yet that if spectral properties of SA are also affected as well under pH conditions, and this needs further investigation.

The application of the ferulic acid esterase or other enzymes which have similar carboxylic acid esterase activity in RSM requires more studies on the characteristics of these enzymes, such as stability during meal processing or in the animal digestive tract environment. The resistance to high temperature pelleting conditions and activity survival in the animal gut may be key factors when considering the enzyme to be used as a feed additive. In addition, enzymatic treatment of feed ingredients in a liquid or high moisture conditions during the processing is not a common practice due to the

increased process (drying) cost. Therefore, attempts must be made to develop a cost-effective procedure or methodology, such as the combination with other anti-nutrient detoxification method, the use of enzyme on the isolated SNP extract, or the application of this enzyme on fractionated meal ingredient.

This new approach on the breakdown pathway of SNP in RSM should be considered to produce SA and choline from isolated SNP and increase the nutritional value of the meal producing the value-added protein products. This goal could be accomplished through taking the advantage of the beneficial effects of SA and choline, which are derived from SNP after enzymatic treatment. This enzymatic technology has the advantage in improving the nutritional value of RSM or CM without nutrient loss, as well can be considered as a major tool in combination with other bio-processing techniques in the removal of anti-nutritional factors or improving nutrient utilization during the meal processing stage under controlled conditions or in the feed.

7.6 Conclusion

Tannase had mild hydrolase activity towards SNP *in vitro,* while tyrosinase, which is a monophenol oxidase, could breakdown both SNP and SA with the possibility of the transformation of these compounds into undesirable quinnone or quinnone derivatives. Phenolase had no any activity on the hydrolysis of SNP.

The ability of ferulic acid esterase to hydrolyze SNP had a broad temperature (50-60°C) and pH range (4 –6) and did not transform SA. Therefore it appears to be a good candidate for the enzymatic hydrolysis of sinapine into sinapic acid and choline in

commercial RSM processing. The novel idea of this enzymatic technology could also be applied to detoxification of anti-nutritional factors in other feedstuffs.

8. GENERAL DISCUSSION

The simple phenolics, SNP and SA, have been considered to be anti-nutritional factors when feeding RSM to non-ruminant animals such as pigs and chickens. For the most part, the evidence for this assessment has been based on *in vitro* research rather than animal feeding trials. The current research is the first to systematically study the effects of purified SNP and SA using sufficient animal numbers (broiler chickens) to allow their evaluation from nutritional, physiological and metabolic points of view (Chapters 4, 5, and 6).

SNP is the predominant simple phenolic in RSM, being commonly found at levels of approximately 1%. Research demonstrated that SNP did not cause obvious anti-nutritional effects in broiler chickens when fed at levels up to those that approximate 30% dietary inclusion of RSM. This statement is based on the lack of a negative effect on growth related production parameters, bird health, and organ or digestive tract size. On the contrary, purified SNP bisulfate trihydrate improved dietary AME, and semi-purified SNP in the form of an ethanol extract improved both AME and apparent protein digestibility, with the largest improvement at the lowest levels of inclusion (0.150 and 0.225 %). The lack of effect on feed intake refutes the claim that SNP reduces the palatability of RSM for broiler chickens, but does not eliminate a possible effect in animals more sensitive to the organoleptic properties of feed.

Free SA is found at relatively low levels in RSM, but is found in larger amounts as a component of SNP. Therefore, it represents an important simple phenolic in RSM

and it is important to understand its nutritional effect(s). As with SNP, low levels of SA did not cause apparent anti-nutritional effects in broiler chickens. Growth, feed efficiency, livability, tissue weights and measurements, and serum CK and LD enzyme activity were unaffected by SA in one trial and in the second trial. SA at the level of 0.025 % stimulated feed intake and increased broiler weight gain in comparison to the control. This level of SA also improved fecal protein digestibility, which is similar to the finding in the SNP trial where the effect may be associated with SA release during SNP hydrolysis. The quadratic effect nature of performance and nutrient digestibility results in both trials suggests that the nutritional benefits derived from simple phenolics are dose dependent and that higher dietary levels will result in little if any response, or possibly negative effects. Additional research on lower levels of dietary SA inclusion is required to confirm the beneficial effects of SA and provide further evidence of its dose dependence.

In terms of the metabolism of SNP and SA in the digestive tract, both similarities and differences were observed. For SNP, ileal digestibility is approximately half the value obtained for fecal digestibility, whereas almost all of the SA was apparently absorbed prior to the ileum. Both SNP and SA were found in excreta, but for SA it appears to mostly be derived from its original form excreted in urine, whereas SNP may come from undigested SNP in the digestive tract or absorbed SNP excreted in urine or both. The finding of SA in both ileum and excreta in SNP bisulfate treatments is the first to disclose that the metabolic pathway of SNP hydrolysis into SA and choline *in vivo* starts prior to the ileum and is not only confined to the hind gut (ceca). SA was consistently absent in the cecal digesta in both SNP and SA trials, and higher SA ileal

digestibility than fecal digestibility, suggest the possibility of transformation of SA prior to or after absorption, or both, with the former possibly due to hind gut bacteria. However, the exact sites and pathways of digestion, absorption and metabolism of these compounds have yet to be determined.

To the author's knowledge, this research is the first to examine the effect of SNP and SA on gut bacterial fermentation and ecology. SNP was found to have no impact on VFA production in the ceca and, therefore, might not have antibacterial activity. In contrast, SA demonstrated a strong inhibitory effect on VFA production, especially acetic acid, in the ceca of broiler chickens and therefore, may have antibacterial activity *in vivo*. Further microbial community analysis based on % G+C indicated a shift in the cecal microbial population rather than a change in the relative abundance of populations within the cecal microbial community. Interestingly, the data showed an increased abundance of bacteria generally considered to be beneficial, and a decreased abundance in pathogenic bacteria. This suggests a microbiologically, as well as a nutritionally, beneficial effect of dietary SA. Therefore, low levels of dietary SA may have potential as a natural growth promotant or an alternative to antibiotics in non-ruminant animals. More research is required to study the mechanism whereby SA inhibits VFA production in the ceca and the effects it has on the microbial community profile. Both SNP and SA increased VFA levels in the ileum but the mechanism or practical significance of these effects remains unknown.

Previous research has demonstrated that a microbial enzyme was capable of reducing the SNP content in RSM, but this research failed to examine the final products of hydrolysis, which likely included relatively toxic quinones instead of choline and the

potentially beneficial SA. Research in this thesis tested the capacity of four microbial enzyme sources to hydrolyze SNP into SA and choline without further modification of SA to quinones. Three of four enzymes were capable of SNP breakdown but one (ferulic acid esterase) demonstrated activity on SNP bisulfate trihydrate as well as SNP in RSM under a broad range of temperature and pH conditions and without modification of SA. A novel concept of this research is based on the premise that SNP hydrolysis to SA and choline would improve the nutritional value of RSM. Research is required to determine whether this could occur in the meal during processing or by adding enzyme to animal diets. Alternately, SNP could be isolated from RSM and hydrolyzed to produce a dietary supplement. The high moisture conditions required for enzyme action are not a normal part of the current RS crushing industry, so enzyme use during processing may not be a realistic practice. More enzyme work needs to be done looking at the addition of ferulic acid esterase or other carboxylic esterases to the formula feed to hydrolyze SNP in the animal's digestive tract.

The UV spectrophotometry quantification methods used in this thesis are sufficient to estimate the total simple phenolics content of RSM, but are not adequate for either individual compound (SNP or SA). This technique may overestimate the actual content of simple phenolics. On the other hand, because of changes in the spectral properties of SNP at different pH conditions, quantification methods based on any techniques may underestimate the SNP content by at least 20 %.

Other aspects of the current research suggest that basic research is still needed to understand the physicochemical properties of simple phenolics and, in turn, how these characteristics relate to the biological response of animals fed these compounds. For

example, SA has been shown to have good antioxidant activity, but there is no information on how it relates to *in vivo* beneficial effects when SA is included in food or feed.

Other findings of this research demonstrate effects of feeding RSM that are not associated with SNP or SA. These include increased serum T3 and T4 hormone levels and proportional liver size. These may be the result of residual glucosinolates in the meal. Fractionation of meal may allow concentration of the responsible agent and permit more detailed study.

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